

UNCLASSIFIED

AD NUMBER
AD445282
NEW LIMITATION CHANGE
TO Approved for public release, distribution unlimited
FROM Distribution authorized to U.S. Gov't. agencies and their contractors; Administrative/Operational Use; JUN 1964. Other requests shall be referred to Walter Reed Army Institute of Research, Washington, DC.
AUTHORITY
USAMRDC 10/5, 19 Jul 1976

THIS PAGE IS UNCLASSIFIED

UNCLASSIFIED

AD 4 4 5 2 8 2

DEFENSE DOCUMENTATION CENTER

FOR

SCIENTIFIC AND TECHNICAL INFORMATION

CAMERON STATION, ALEXANDRIA, VIRGINIA



UNCLASSIFIED

NOTICE: When government or other drawings, specifications or other data are used for any purpose other than in connection with a definitely related government procurement operation, the U. S. Government thereby incurs no responsibility, nor any obligation whatsoever; and the fact that the Government may have formulated, furnished, or in any way supplied the said drawings, specifications, or other data is not to be regarded by implication or otherwise as in any manner licensing the holder or any other person or corporation, or conveying any rights or permission to manufacture, use or sell any patented invention that may in any way be related thereto.

445282

CATALOGED BY DDC

AS AD NO. _____

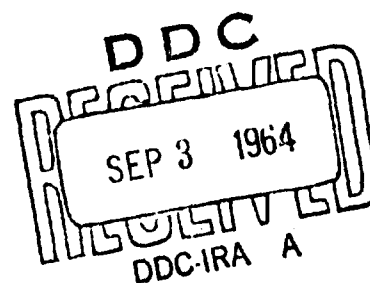
WALTER REED ARMY INSTITUTE OF RESEARCH
WALTER REED ARMY MEDICAL CENTER
WASHINGTON, D.C. 20012

ANNUAL PROGRESS REPORT



Reports Control Symbol MEDDH-288

1 July 1963 - 30 June 1964



Volume II

Qualified requestors may obtain copies of this report from DDC.

This Document Contains
Missing Page/s That Are
Unavailable In The
Original Document

Best Available Copy

WALTER REED ARMY INSTITUTE OF RESEARCH

Walter Reed Army Medical Center

Washington, D. C. 20012

ANNUAL PROGRESS REPORT

1 July 1963 -- 30 June 1964

Volume II

Reports Control Symbol MEDDH-288

Qualified requestors may obtain copies of this report from
the Defense Documentation Center

In conducting the research described in this report, the investigators adhered to the "Principles of Laboratory Animal Care as established by the National Society for Medical Research."

ARMY RESEARCH TASK REPORT		REPORTS CONTROL SYMBOL CSCRD-6(R2)
ACCESSION NUMBER 36165	PROJECT, TASK, OR SUBTASK NO. 3A012501B8130201	
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D. C., 20315	2. FUNDING AGENCY Army Medical R&D Command Office of The Surgeon General Washington, D. C., 20315	
3. CONTRACTING AGENCY NA	4. CONTRACTOR AND/OR GOV'T LABORATORY A Walter Reed Army Inst of Rsch Walter Reed Army Medical Center Washington, D. C., 20012 723-1000, Ext 3552	
5. PRINCIPAL & ASSOC. INVESTIGATOR/PROJECT OR ACTION OFFICER (P) Hahn, Fred E., Ph.D., Dept of Molecular Biology, Div of Comm Dis and Immunol, WRAIR, WRAMC, Washington, D. C., 20012 576-3657 or Interdepartmental Code 198, Ext 3657 See Continuation Sheet		
6. TITLE OF PROJECT <input type="checkbox"/> <div style="display: flex; justify-content: space-between;"> <div> TASK <input type="checkbox"/> SUBTASK <input type="checkbox"/> </div> <div>Mode of action of antimicrobial agents (U)</div> </div>		
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964		
8. RESUME (U) The antimalarial drug, chloroquine, was found also to be a very strong antibacterial agent. It interacts with DNA presumably by interposition between the planes of the hydrogen-bonded purine and pyrimidine bases. Erythromycin inhibits a cell-free ribosomal model system of protein synthesis that synthesizes polyphenyl-alanine. Marked accumulation of transfer-RNA in streptomycin-exposed bacteria is accompanied by a dramatic decline in ribosomal RNA synthesis; this is attributed to a progressive inhibition of RNA polymerase by transfer-RNA. Chloramphenicol inhibits protein synthesis by interfering with the formation of the messenger RNA-ribosome complex through direct interaction with ribosomes. Kinetic studies show that the effect of chloramphenicol upon the ribosome-poly U-system is reversible and satisfied formal criteria for competitive inhibition. Hydrolysates of bacterial DNA contain >15 amino acids amounting to 0.1% of the mass of DNA. Presumably, these amino acids are derived from an "initiator protein" responsible for triggering individual rounds of replication of the genome. In order to visualize some of the macromolecular events cited above, a modified vacuum system has been developed for the electron microscope which eliminates object contamination and allows the utilization of the full resolution power of the instrument.		
9. KEY WORDS Molecular biology, antimicrobial agents, chemotherapy, proteins, nucleic acids, control mechanism, gene replication, biochemistry, biophysics, malaria		
10. SUPPORTING PROJECTS <div style="text-align: center;">Not Applicable</div>		
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet	12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet	
<div style="display: flex; justify-content: space-between;"> <div>DA FORM 1309R 1 June 60</div> <div>PREVIOUS EDITIONS ARE OBSOLETE</div> <div>PAGE 1 of 1</div> </div>		

ACCESSION NO.
36165

ARMY RESEARCH TASK REPORT

13. PROJECT, TASK OR
SUBTASK NUMBER

11 22 23 24 25 26 27 28 29
3 A 0 1 2 5 0 1 B 8 1 3 0 2 0 1

14. DATE OF REPORT (30-33)

30 33 34
0 6 6 4 4

15. SECURITY OF WORK (34)

16. TYPE OF REPORT

35 36 47 48 49 50 51 52 55
3 1 2 6 3

17. SCIENTIFIC FIELD

a. Topical Classific. (56-61)
b. Functional Class (62-64)

56 61 62 64
0 1 0 2 0 7

18. OSD CLASSIFICATION

(65-66)

19. R&D CATEGORY (67)

65 66 67
B R 1

20. CONTRACT NUMBER

11 12 13 14 15 17 18 21 22 26 27
D A

21. GRANT NUMBER

28 29 30 33 34 35 36 38 39 40 41 45 46
D A G

22. ESTIMATED COMPLET.
DATES

47 51 52 56 57 61 62 66 67 71
1 C O N T 2 3 4 5

23. PRIORITY (11-14)

24. PROGRAM ELEMENT
(15-26)

11 14 15 26
2 6 • 1 1 • 2 5 • 0 1 • 1

25. CMR&D CODES

27 29 30 32 33 35
N / A

26. CDOG REFERENCE

a. Paragraph No. (36-44)

b. Functional Group (45)

36 39 40 41 42 43 44 45
1 4 1 2 a 6

27. FUNDING

a. Est. Total Cost (11-15)

b. % Spent Intern. (16-18)

" " Extern. (19-21)

c. Total Obligation (22-26)

d. Progrmd. Cur. FY (27-33)

e. " " " +1 (34-40)

f. " " " +2 (41-47)

g. " " " +3 (48-54)

h. " " " +4 (55-61)

i. " " " +5 (62-68)

j. " " " +6 (69-75)

k. Total Man Years of
Effort (76-78)

11 15 16 18 19 21 22 26
1 2
27 28 29 33 34 35 36 40
41 42 43 47 48 49 50 54
55 56 57 61 62 63 64 68
69 70 71 75 76 78

DA FORM 1309R
1 June 63

Previous Editions are Obsolete

Page 2 of

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

- (A) Allison, J. L., Capt, MSC, Dept of Molecular Biology
Div of Comm Dis & Imm, WRAIR, WRAMC, Washington, D. C., 20012
576-3435 or Interdepartmental Code 198, Ext 3435 49
- (A) Ciak, J., M.S., Dept of Molecular Biology
Div of Comm Dis & Imm, WRAIR, WRAMC, Washington, D. C., 20012
576-3657 or Interdepartmental Code 198, Ext 3657 49
- (A) Hartman, R. E., Ph.D., Dept of Molecular Biology
Div of Comm Dis & Imm, WRAIR, WRAMC, Washington, D. C., 20012
576-3435 or Interdepartmental Code 198, Ext 3435 49
- (A) Hartman, R. S., Ph.D., Dept of Molecular Biology
Div of Comm Dis & Imm, WRAIR, WRAMC, Washington, D. C., 20012
576-3435 or Interdepartmental Code 198, Ext 3435 49
- (A) Olenick, J. G., M.S., Dept of Molecular Biology
Div of Comm Dis & Imm, WRAIR, WRAMC, Washington, D. C., 20012
576-3657 or Interdepartmental Code 198, Ext 3657 49
- (A) Wolfe, A. D., S.M., Dept of Molecular Biology
Div of Comm Dis & Imm, WRAIR, WRAMC, Washington, D. C., 20012
576-3657 or Interdepartmental Code 198, Ext 3657 49

REPORTS. Annual Progress Report, Walter Reed Army Institute of Research, 1 July 1963 - 30 June 1964.

Hahn, F. E., Action des Antibiotiques sur la Synthèse des Protéines. *Médecine et Hygiène*, 22:341, 1964.

Hahn, F. E., On Traditional and Modern Biology. *Science*, 141:1525, 1963.

Hahn, F. E., Biology and Metaphysics. *Science*, 142:15125, 1963.

Wolfe, A. D. and Hahn, F. E., Erythromycin: Mode of Action. *Science*, 143:1445, 1964.

Ciak, J. and Hahn, F. E., On Nucleic Acids Accumulating Under the Influence of Streptomycin in *Escherichia coli*. *Bact. Proc.*, 1964, p 98.

Wolfe, A. D. and Hahn, F. E., Studies on Chloramphenicol, Ribosomes, and an Amino Acid Incorporating System of *E. coli* Origin. *Fed. Proc.*, 23:269, 1964.

ANNUAL PROGRESS REPORT

Project No. 3A012501B813

Title: ARMY MEDICAL BASIC RESEARCH
IN LIFE SCIENCES

Task No. 02

Title: Microbiology

Subtask No. 01

Title: Mode of action of anti-
microbial agents

Description:

A study of the molecular biology, biochemistry and microbiology of bacteria under the influence of antimicrobial agents in order to elucidate the modes and mechanisms of action of these agents.

Progress:

Mode of Action of Chloroquine.

The emergence of chloroquine resistance in several strains of Plasmodium falciparum has become a matter of grave concern to AMEDS. In order to understand the nature of this resistance phenomenon, it is essential to elucidate the mode of action of chloroquine, as well as to study its potential role as a mutagenic agent.

The mode of action of chloroquine is unknown. Since the drug is equally active in vitro and in vivo (Taylor et al., Am. J. Trop. Med. Hyg. 1:132, 1952) its molecule does not require conversion in vivo into some form of "active metabolite." Two observations appear to be pertinent to the problem of the mode of action of chloroquine. Parker and Irvin (J. Biol. Chem. 199:897, 1952) have demonstrated by spectrophotometry and viscosimetry an interaction of chloroquine with nucleic acids, especially with DNA, and Schellenberg and Coatney (Biochem. Pharmacol. 6:143, 1961) have shown that chloroquine interfered with the biosynthesis of plasmodial nucleic acids. Since this effect requires drug (molar) concentrations one order of magnitude higher than those of cinchona alkaloids or mepacrine, producing the same inhibition, the authors concluded that the effects on nucleic acid synthesis were "unrelated" to the mode of action of chloroquine.

The essential precondition for successful studies on modes of action is the availability of a sensitive test organism that is readily grown in quantity and lends itself to the application of methods of molecular biology. It is a scientific fallacy to assume that the study of the mechanism of action of an antimalarial drug requires the use of plasmodia as test organisms. Any bacterium that is subject to growth inhibition by chloroquine at a concentration range of 10^{-5} M appears to be suitable.

In a first series of experiments it was shown readily that several bacterial species (Sarcina lutea, Bacillus Subtilis, Micrococcus lysodeicticus) are highly sensitive to chloroquine. Strains of E. coli were less sensitive but exhibited characteristic changes in the morphology of their chromatin material upon exposure to chloroquine. Since B. subtilis is a favorable organism for precision studies on type transformation and M. lysodeicticus lends itself ideally to the preparation of cell-free extracts and constituents, the technical preconditions for studies on the mode of action of chloroquine now exist. A few preliminary attempts at demonstrating a mutagenic effect of the drug have been unsuccessful but efforts in this direction continue. Experiments are also under way to determine if any of the essential macromolecular syntheses (protein, DNA, RNA) are specifically inhibited by chloroquine.

Based on the earlier work of Parker and Irvin, loc. cit., experiments have been conducted to study the nature of the interaction between chloroquine and DNA. Highly purified DNA from E. coli was used. It was readily demonstrated that the absorption spectrum of chloroquine is very markedly altered in the presence of DNA while the spectrum of DNA shows no appreciable change. These findings indicate that chloroquine interacts with DNA but they do not specify the nature of the interaction.

The hypothesis has been proposed (F. E. Hahn, on Chemotherapy of Malaria, Report to the Director, WRAIR, December 1963) that by virtue of its molecular dimension, shape and charge distribution, chloroquine might be interposed between the planes of base pairs in the DNA double helix. Separation of the two strands of native DNA can be experimentally demonstrated by following the changes in absorbancy at 260 m μ in solutions of DNA that are being heated through a temperature interval. Strand separation, and hence the establishment of a greater degree of randomness results in increases in absorbancy at 260 m μ of the order of 40 per cent. The specific temperature at which one half of this change is manifest is called the T_m temperature. Under standard experimental conditions, the value of T_m is a natural constant for each DNA of a given base composition. It provides a measure of the overall strength with which the two companion strands of DNA are held together by hydrogen bonds. The T_m temperature of DNA in the presence of a few micrograms of chloroquine was elevated by 10° C, i. e., by a very large temperature interval. This observation suggests that the hypothesis mentioned above is correct and that chloroquine contributed to the overall strength with which the two companion helices hold together. It suggests further that chloroquine might interfere with the normal replication of the genomes of sensitive organisms and raises a strong suspicion that the drug is mutagenic. Detailed biophysical studies on the interaction between chloroquine and DNA continue.

Mode of Action of Erythromycin.

It has been known for some time that the antibiotic, erythromycin, inhibits the biosynthesis of protein in susceptible bacteria but permits continued synthesis of nucleic acids. The action of erythromycin can, thus, be compared to that of chloramphenicol, and the two drugs inhibit bacterial growth in an additive manner (Brock and Brock, *Biochim. Biophys. Acta.* 33:274, 1959).

Antibiotics which are specific inhibitors of protein synthesis in vivo have been shown to inhibit the incorporation of radioactive amino acids into trichloroacetic acid-insoluble polymers by cell-free ribosomal systems prepared from sensitive bacteria. This has been demonstrated by various authors for chloramphenicol, puromycin, tetracyclines and streptomycin. Inhibition of such cell-free systems by inhibitors of protein synthesis, especially chloramphenicol, is now generally regarded as circumstantial evidence that such a system represents a valid model system for protein synthesis.

A cell-free ribosomal system was prepared after the methods of Nirenberg and Matthaei (*Proc. Nat. Acad. Sci.* 47:1588, 1961), using E. coli as a source of ribosomes and soluble factors. In such a system, erythromycin inhibited the formation of polyphenylalanine, under the direction of polyuridylic acid as messenger-RNA, from added radioactive phenylalanine. The data are listed in Table I.

TABLE I

Additions	Radioactivity in polymer (counts per min)
Complete System	33,649
Energy source (ATP) omitted	2,762
Messenger-RNA (poly U) omitted	764
1.0 μ mole Erythromycin added	14,815
1.5 μ mole Erythromycin added	6,765

The detailed mechanism at the molecular level by which erythromycin inhibits protein synthesis is still unknown. The antibiotic is a member of the macrolide group. It contains a 14-membered ring system of aliphatic carbon atoms closed by a lactone oxygen bridge. The two sugars, desosamine and cladinose, are substituted to this ring system. The chemical structure of erythromycin offers no ready clue to its mechanism of action.

It was observed in this laboratory, however, that erythromycin is capable of precipitating RNA and certain synthetic polynucleotides which can serve as models of messenger-RNA in cell-free ribosomal systems. It is inferred that the mechanism of action of erythromycin may involve an interaction with one or several categories of RNA that mediate protein biosynthesis. By virtue of possessing a considerable number of hydroxyl groups, erythromycin should be able to engage in multiple hydrogen bond formation.

A preliminary report by Vasquez (Biochem. Biophys. Res. Comm. 12:409, 1963) that erythromycin prevents the attachment of radioactive chloramphenicol to bacterial ribosomes has been readily confirmed in this laboratory and expanded to the demonstration that this effect of erythromycin is reversible and competitive in nature. It is plausible, therefore, to assume that the sites of action of erythromycin and of chloramphenicol on the ribosome are identical.

Mode of Action of Streptomycin

It was originally discovered in this laboratory that streptomycin inhibits protein synthesis in susceptible bacteria (Hahn and Ciak, Bact. Proc. 1959, p. 131). This view has gained general acceptance, especially after it was shown by other investigators that the antibiotic also inhibits protein synthesis in cell-free model systems.

One part of the original findings in this laboratory consisted of the demonstration that in streptomycin-exposed bacteria the biosynthesis of nucleic acids continued for approximately one generation time but eventually became minimal. The nature of nucleic acids synthesized during streptomycin action and the reason for the rapid decline in the rate of nucleic acid synthesis have been subject of intensive investigation in this laboratory. In the preceding Annual Report, some results of centrifugal studies were reported which suggested that the synthesis of ribosomal RNA, the bulk of bacterial RNA, declines rapidly in streptomycin action and that a low-molecular RNA with the sedimentation properties of transfer RNA accumulates.

A conclusive study of this problem has now been carried out. A special chromatographic column, consisting of methylated albumin adsorbed onto kieselguhr (henceforth referred to as MAK column) was employed (Mandell and Hershey, Analyt. Biochem. 1:66, 1960). The MAK column separates nucleic acids upon elution with a linear gradient of NaCl solution into the transfer-RNA fraction, the DNA, and the 16 s and 23 s ribosomal RNAs.

Cultures of E. coli in a synthetic medium were exposed to 30 μ g/ml of streptomycin for 60 min. These cells and cells from a control culture without streptomycin were collected, washed and lysed by a sequence of lysozyme and detergent treatments. The

nucleic acids were extracted from the lysates with phenol. Overall nucleic acid concentrations were standardized spectrophotometrically, and identical numbers of adsorption units (260 mμ) were chromatographed on a MAK column. The nucleic acids from the streptomycin-exposed culture showed a markedly different distribution from that of the normal bacterial nucleic acids. There was a huge excess of transfer-RNA and correspondingly much less ribosomal RNA present in streptomycin-treated bacteria,

When an excess of radioactive uracil was supplied to the experimental culture immediately after the addition of streptomycin, all nucleic acids produced during streptomycin action were labelled. The net analytical increase in RNA during one hour of streptomycin action was 66 per cent. The fractions collected from the eluate of the MAK column were subjected to liquid scintillation counting, and the distribution of radioactivities was made commensurate to the distribution of nucleic acid mass and compared. The overwhelming amount of RNA produced during the action of streptomycin was found in the transfer-RNA fraction. Little radioactive material was in the ribosomal RNA fraction, and the chromatographic distribution of this material differed so markedly from that of normal bacterial ribosomal RNA that it represents probably a collection of different molecular species which are either precursors or degradation products of ribosomal RNA. No radioactive material was eluted beyond the position of the 23 s ribosomal RNA suggesting that messenger-RNA did not accumulate to a significant extent during streptomycin action.

The streptomycin "transfer-RNA" was isolated and tested for its ability to react with activating enzymes, ATP, and amino acids, i.e., for its actual function as transfer-RNA. Five amino acids were tested and for all of them the specific acceptor activity of normal transfer-RNA and streptomycin transfer-RNA was identical within the limits of error. It must be concluded that the streptomycin material chromatographing in the transfer-RNA region is normal competent transfer-RNA.

Tissi eres et al., (J. Molec. Biol. 7:100, 1963) have shown that the enzyme RNA polymerase which is responsible for the transcription of RNA from DNA is strongly inhibited by free transfer-RNA and appreciably by amino acyl-charged transfer-RNA. The present findings can be explained by assuming that the large quantities of transfer-RNA formed in streptomycin-treated bacteria inhibit progressively the RNA polymerase and thus throttle the synthesis of ribosomal RNA. This work is nearly completed and a publication is in preparation. The problem of the mechanism of action of streptomycin, however, still awaits solution. This is likely to be accomplished when the structure and function of the ribosomal particles become more completely understood.

Mode of Action of Chloramphenicol.

Chloramphenicol acts in an area of protein synthesis beyond the synthesis of amino acyl transfer-RNAs but prior to the formation of peptide bonds between amino acids. Two types of processes take place in this area: (1) the assemblage of complexes of ribosomes and messenger-RNA (polysomes) and (2) enzymatic processes by which amino acyl transfer-RNAs are linked to the 50 s moiety of ribosomal particles and the amino acids are condensed with the formation of peptide bonds.

Chloramphenicol resistance of mutants from chloramphenicol-sensitive wild strains of bacteria is a polygenic cooperative phenomenon. This makes it appear unlikely that the action of the antibiotic is upon a single enzyme, the mutational change of which would lead to strong drug resistance in one single mutational step. This genetic argument focuses the attention upon macromolecular assemblages as the processes most likely to be inhibited by chloramphenicol.

A suggestion that such might be the case was forwarded by Jardetzky and Julian (Nature 201:397, 1964) who have shown that chloramphenicol prevented partly the formation of polysomes from ribosomes and radioactive polyuridylic acid (as a model for messenger RNA). Since a poly U-instructed cell-free ribosomal system which synthesizes polyphenylalanine (see section on Mode of Action of Erythromycin) is relatively insensitive to inhibition by chloramphenicol ($2 \times 10^{-3}M$ required for partial inhibition) the use of poly U in Jardetzky's experiments was a somewhat unfortunate choice. A ribosomal system with polyadenylic acid (poly A) synthesizes polylysine; this system is highly sensitive to the action of chloramphenicol (Speyer et al., Cold Spring Harbor Symp. 28:559, 1963). Preliminary experiments in this laboratory have shown that chloramphenicol suppresses the formation of a complex of ribosomes and poly A completely. It is inferred that the antibiotic inhibits protein synthesis by preventing the essential interaction between ribosomes and messenger-RNA. This idea also explains the selective toxicity of chloramphenicol. Microbial protein synthesis is mediated by ad hoc assemblages of ribosomes and messenger-RNA and is sensitive to the action of the antibiotic; mammalian protein synthesis is mediated by more permanent combinations of ribosomes and messenger-RNA that is not subject to turnover: this type of protein synthesis is insensitive to chloramphenicol.

An interference with the formation of ribosome-messenger-RNA complexes by chloramphenicol might logically be brought about by an interaction of the antibiotic with either ribosomes or messenger-RNA. Studies using radioactive chloramphenicol in this laboratory have shown that the site of action of chloramphenicol is on the ribosome. Purified ribosomes of E. coli were suspended in solutions of radiochloramphenicol in a series of graded specific radioactivities. These suspensions were sedimented by preparative ultracentri-

fugation, the sedimented ribosomes were resuspended and subjected to liquid scintillation counting for carbon-14. The amount of radio-chloramphenicol associated with ribosomes was strictly proportional to (1) ribosomal mass, and (2) specific radioactivity of the suspending chloramphenicol solution. This proves the existence of competition between C-12 and C-14 chloramphenicols for ribosomal binding sites. Had the ribosomal sediments merely entrained a given volume of chloramphenicol solution without specific binding, the counted radioactivities would have also been proportional to ribosomal mass but would have been identical for all graded solutions of chloramphenicol containing the same total radioactivity, irrespective of the differences in specific radioactivities. Efforts at demonstrating binding of chloramphenicol to RNA and to synthetic polynucleotides were unsuccessful. It is concluded that chloramphenicol prevents the attachment of messenger-RNA to ribosomes by interacting with the ribosomes.

Since the interaction of messenger-RNA with ribosomes, the interaction of messenger-RNA with amino acyl transfer-RNA (i.e., the actual reading of the genetic code), and the initial (non-enzymatic) interaction of amino acyl transfer-RNA with the 50 s moiety of ribosomes are relatively weak and reversible processes, one should expect that the effect of chloramphenicol (which is a bacteriostatic, hence a reversible effect) should also be reversible when studied in cell-free model system of protein synthesis. This was demonstrated in this laboratory using a general experimental design outlined by Ackermann and Potter (Proc. Soc. Exp. Biol. Med. 72:1, 1949). In the ribosomal poly U system which synthesizes polyphenylalanine (see section on Mode of Action of Erythromycin) concentrations of either, ribosomes, or poly U, or transfer-RNA were made the rate limiting factors for the initial velocities of formation of radioactive polyphenylalanine. When these concentrations were graded, it was readily shown that within certain concentration limits the reaction velocities were directly proportional to concentrations of ribosomes, poly U, or transfer-RNA. The presence of a constant amount of chloramphenicol reduced the reaction velocities by constant percentages rather than by constant amounts. This type of finding is indicative of reversible inhibition.

When the data from these kinetic experiments were evaluated by plotting the reciprocals of the rate-limiting concentrations of ribosomes, poly U, or transfer-RNA as a function of the reciprocals of the initial reaction velocities carrying out this data reduction for control experiments and for experiments with partly inhibitory concentrations of chloramphenicol, typical Lineweaver-Burke-plots were obtained in which the lines representing the control experiments and the inhibition experiments intercepted on the ordinate. It is customary to consider this type of result as evidence for competitive inhibition. Rather than assuming that all three components (ribosomes, poly U, transfer-RNA) are competitors of chloramphenicol in the assemblage of a functional ribosomal system,

it is thought that the data probably reflect a kinetic situation which is too complicated for a superficial application of the double reciprocal plot.

The problem of the mechanism of action of chloramphenicol has been reduced to the same set of questions that arise in connection with studies on streptomycin, erythromycin and tetracyclines, viz., these of the nature of the structure and function of the ribosomal particles as production units in protein synthesis.

Effects of Inhibitors of protein Synthesis on DNA Synthesis.

Inhibition of protein synthesis in microorganisms, for example by chloramphenicol, erythromycin, streptomycin and tetracyclines, permits continued synthesis of DNA. This DNA synthesis, however, does not usually attain increments of 100 per cent before it comes to a standstill. Studies by Maaløe and his associates (J. Cell. Comp. Phys. 62 suppl. 31, 1963) have shown that the replication of the genome in bacteria occurs in cycles and that some form of protein synthesis is required to initiate each new cycle. In the absence of protein synthesis, therefore, a typical asynchronous culture permits those organisms to complete the cycle which have already initiated it at the time when protein synthesis becomes inhibited.

The nature of this "imitator protein" is hypothetical (Jacob et al., Cold Spring Harbor Symp. 28:329, 1963). It may be a soluble enzyme or could be a protein that becomes actually attached to completed DNA at the beginning of another replication cycle.

Associations of DNA with proteins, peptides, or amino acids, have been demonstrated for mammalian DNA and phage DNA under conditions which make it appear extremely unlikely that these materials represent residual contaminations carried over from the preparation of DNA. No such demonstration had been made so far for bacterial DNA. Studies in this laboratory have now revealed the presence in E. coli K-12 DNA hydrolysates of 15 different amino acids that jointly constitute about 0.1 per cent of the mass of DNA.

DNA was prepared from mass cultures of E. coli K-12 by a modification of the procedure of Marmur (J. Mol. Biol. 3:208, 1961) by increasing the number of deproteinization steps, including several phenol extractions, and increasing the number of reisolutions of DNA to such an extent that it virtually eliminates any possibility of carrying contaminating bacterial proteins over into the final DNA preparation.

This DNA was native, i. e., double-stranded since it exhibited a hyperchromic shift at 260 m μ of 40 per cent upon heating through a temperature interval from 80 - 96° C and had a T_m temperature of 88 - 90° C, typical for E. coli DNA. The sedimentation coefficient of this material was 18-20 s, indicating that the DNA prep-

ration was of high molecular weight.

Paper chromatography of acid hydrolysates of this DNA revealed the presence of glycine and ammonia. These substances represented degradation products of the constituent bases of DNA as they were also found in solutions of individual purine and pyrimidine bases or deoxyribosides that had been subjected to the conditions of acid hydrolysis.

Automatic amino acid analysis of hydrolysates of 300 mg samples of DNA resulted in the identification and determination of 15 different amino acids listed in Table II. Milligram quantities of glycine and ammonia were not tabulated nor considered in the interpretation of data. Alanine could not be determined because the large glycine peak eclipsed the elution area of that amino acid. Columns 5 and 6 in Table II list the moles per cent for the amino acids determined in samples 1a and 2; columns 7 and 8 list the corresponding numbers calculated for the same amino acid population of 15 from the analyses of the proteins of *E. coli* of Roberts *et al.* (Studies of Biosynthesis in *Escherichia coli*, Carnegie Inst. of Washington, Washington, D.C., 1955, p 28) and of Sueoka (Proc. Nat. Acad. Sci. 47:1141, 1961).

The relative abundance of amino acids in the hydrolysates of *E. coli* DNA was different from the global amino acid composition of the bacterial proteins. This appears to render unlikely the possibility that these amino acids were derived from the hydrolysis of small quantities of bacterial proteins, contaminating the DNA preparations *at random*. The nature of the association of these amino acids, suggesting the presence of a protein bound to DNA, is under investigation.

Influence of antimicrobial agents upon the normal replication of the genome, be it direct as assumed for chloroquine, or indirect as postulated for the inhibitors of protein synthesis, requires verification by electron microscopy. This type of study must utilize the highest magnification and full resolution power of modern electron microscopes. These instruments resolve objects two to five Ångström units apart; however, the amount of information obtainable at this level of resolution has remained limited because of the rapid accumulation of large quantities of contaminating materials on the specimen.

Most modern electron microscopes operate at pressures of the order of 10^{-4} Torr; at these pressures, the partial pressures of residual hydrocarbons are of the order of 10^{-5} to 10^{-6} Torr. Consequently, monomolecular layers of hydrocarbon form on all inner surfaces of the instrument in equilibrium with the gas phase. This occurrence would be of little consequence except that at critical areas in the microscope where the electron beam strikes, the molecules of the monolayer become activated and polymerize to

TABLE II

AMINO ACIDS IN DNA AND IN PROTEINS OF *ESCHERICHIA COLI*

	Sample		Sample		Sample		mole % in <i>E. coli</i> protein	
	1 (micrograms)	2	1 (moles X 10 ³)	2	1 (mole%)	2	Roberts et al., 1955	Sueoka, 1961
Arg	26.1	9.6	1.77	0.63	5.0	3.3	6.8	9.2
Asp	47.2	29.8	3.55	2.26	10.0	11.3	12.6	12.6
Cys	22.7	23.2	1.89	1.93	5.3	9.7	2.2	0.5
Glu	130.0	53.6	8.83	3.66	24.8	18.3	13.4	13.7
His	26.8	17.0	1.73	1.10	4.8	3.5	1.2	2.4
Ileu	34.5	16.5	2.03	1.28	7.4	6.3	3.9	6.6
Leu	32.1	19.1	2.45	1.46	6.9	7.3	10.1	10.4
Lys	75.0	40.1	5.13	2.36	14.4	13.8	8.9	7.8
Met	5.9	4.1	0.39	0.41	1.1	2.1	4.3	3.4
Phe	16.0	4.6	0.97	0.23	2.7	1.4	4.2	4.2
Pro	trace	trace	-	-	trace	trace	5.9	5.0
Ser	19.3	11.2	1.36	1.52	5.2	7.7	7.8	5.8
Thr	17.6	13.4	1.48	1.29	4.1	6.5	7.0	5.8
Tyr	10.7	trace	0.59	-	1.6	trace	2.7	3.4
Val	22.1	15.6	2.00	1.33	6.7	6.7	7.0	9.1

form nonvolatile layers of contamination.

The most critical areas of this type are the specimen itself, as well as the condensor and objective apertures. The accumulation of contamination on the specimen occurs at a rate of several hundred Angströms per min, i.e., after only one minute the specimen is covered with a layer of contaminating material 100 times as thick as the structures which the microscopist wishes to resolve. Additionally, the usually asymmetrical contamination of the aperture results in the introduction of asymmetry of the electrical lenses at critical points in the instrument; this produces a rapid deterioration of the quality of the electron image.

It is, therefore, essential for electron microscopy of objects of molecular dimensions that contamination is eliminated. During the past year a number of pilot studies have been conducted from which sufficient data have been obtained to show that it is possible to operate an electron microscope at pressures approaching 10^{-8} Torr with hydrocarbon partial pressures well below this figure. The important changes which are necessary to achieve this include: (1) replacement of all gasketing material with either "Viton A," a material having extremely low vapor pressure and low porosity, or with metal gaskets, (2) elimination of all leaks to a level where the system has the necessary vacuum integrity to operate at these pressures, and (3) the replacement of the usual oil diffusion pumps with a high-velocity titanium "getter-ion" pump. The final pilot study succeeded in pumping the instrument clean and preventing contamination of all critical surfaces. Specimen contamination rates were reduced from several hundred Angströms per minute to less than one Angström, and contamination of the aperture has been eliminated.

In this final study, a temporary arrangement was used which had the disadvantage that pump-down times during routine operation were longer than practical. The data from this study, however, has led to the design of a system to be incorporated into the Siemens Elmiskop I which provides for satisfactory routine operation and will eliminate contamination of all critical areas. The permanent modification of the Siemens Instrument in the Department of Molecular Biology is under way.

Summary and Conclusions:

The antimalarial drug, chloroquine, was found also to be a very strong antibacterial agent. It interacts with DNA presumably by interposition between the planes of the hydrogen-bonded purine and pyrimidine bases. Erythromycin inhibits a cell-free ribosomal model system of protein synthesis that synthesizes polypeptidylalanine. Marked accumulation of transfer-RNA in streptomycin-exposed bacteria is accompanied by a dramatic decline in ribosomal RNA synthesis; this is attributed to a progressive inhibition of

RNA polymerase by transfer-RNA. Chloramphenicol inhibits protein synthesis by interfering with the formation of the messenger RNA-ribosome complex through direct interaction with ribosomes. Kinetic studies show that the effect of chloramphenicol upon the ribosome-poly U-system is reversible and satisfied formal criteria for competitive inhibition. Hydrolysates of bacterial DNA contain >15 amino acids amounting to 0.1% of the mass of DNA. Presumably, these amino acids are derived from an "initiator protein" responsible for triggering individual rounds of replication of the genome. In order to visualize some of the macromolecular events cited above, a modified vacuum system has been developed for the electron microscope which eliminates object contamination and allows the utilization of the full resolution power of the instrument.

ARMY RESEARCH TASK REPORT		REPORTS CONTROL SYMBOL CSCRD-6(R2)
ACCESSION NUMBER <div style="text-align: right; margin-right: 50px;">36166</div>	PROJECT, TASK, OR SUBTASK NO. <div style="text-align: right;">3A012501B8130202</div>	
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D.C. 20315	2. FUNDING AGENCY Army Medical R&D Command Office of The Surgeon General Washington, D.C. 20315	
3. CONTRACTING AGENCY <div style="text-align: center;">NA</div>	4. CONTRACTOR AND/OR GOV'T LABORATORY A Walter Reed Army Inst of Rsch Walter Reed Army Medical Center Washington, D.C., 20012 723-1000, Ext 3552 <div style="text-align: right; border: 1px solid black; width: 20px; float: right;">49</div>	
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Baron, L.S., Ph.D., Dept of Bacterial Immunology, Division of Communicable Disease and Immunology, WRAIR, WRAMC, Washington, D.C. 20012 Sheet 576-2230 or Interdepartmental Code 198, Ext 2230. See Continuation/ 49		
6. TITLE OF: PROJECT <input type="checkbox"/> TASK <input type="checkbox"/> SUBTASK <input checked="" type="checkbox"/> Microbial genetics and taxonomy (U)		
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964		
8. RESUME' (U) R-factors, consisting of an episome, RTF, and carrying drug resistance markers may be transmitted among enteric bacteria by conjugation. The deoxyribonucleic acid of R-factors may be isolated after transmission into species of Proteus. The relative size and molecular makeup of different R-factors have been examined. An unusual Proteus strain was examined and found to contain an infectious element, an episome, which causes the cells to produce the enzymes for lactose utilization. This element and the enzymes were examined. Interrupted mating experiments with an Hfr strain of <u>Salmonella typhosa</u> and an Hfr strain of <u>S. typhimurium</u> have shown that these <u>Salmonella</u> donors transfer their chromosome to <u>S. typhimurium</u> recipients at a rate which is only 0.63 that of an Hfr strain of <u>Escherichia coli</u> to <u>E. coli</u> recipients. The genetic basis of mouse virulence was investigated with an avirulent <u>Salmonella</u> as donor and a virulent <u>S. typhimurium</u> as recipient in recombination experiments. The transfer of partial avirulence was found to segregate among the hybrids. Two genetic determinants were postulated as accounting for the partial avirulence of the hybrids. Both determinants were essential for the phenotypic expression of complete avirulence.		
9. KEY WORDS <div style="text-align: center;">Genetics, salmonella, episomes, virulence, enteric</div>		
10. SUPPORTING PROJECTS <div style="text-align: center;">Not Applicable</div>		
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <div style="display: flex; justify-content: space-between;"> <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet </div>	12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES <div style="display: flex; justify-content: space-between;"> <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet </div>	
<div style="display: flex; justify-content: space-between;"> <div> FORM DA 1 June 63 1309R </div> <div>PREVIOUS EDITIONS ARE OBSOLETE</div> <div> PAGE 1 of ____ </div> </div>		

ACCESSION NO.

36166

ARMY RESEARCH TASK REPORT

13. PROJECT, TASK OR
SUBTASK NUMBER

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
2	A	0	1	2	5	0	1	B	3	1	2	0	2	0	2			

14. DATE OF REPORT (30-33)

30	31	32	33	34
0	6	6	4	4

15. SECURITY OF WORK (34)

16. TYPE OF REPORT

35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55
3																	1	2	6	3

17. SCIENTIFIC FIELD

a. Topical Classific. (56-61)

b. Functional Class (62-64)

56	57	58	59	60	61	62	63	64
0	1	0	2	0	4			

18. OSD CLASSIFICATION

(65-66)

19. R&D CATEGORY (67)

65	66	67
3	R	1

20. CONTRACT NUMBER

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
D	A															

21. GRANT NUMBER

28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46
D	A												G					

22. ESTIMATED COMPLET.
DATES

47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71
1	C	C	N	T	2					3					4					5				

23. PRIORITY (11-14)

24. PROGRAM ELEMENT
(15-26)

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
			2	6	.	1	1	.	2	5	.	0	1	.	1

25. CMR&D CODES

27	28	29	30	31	32	33	34	35
N	/	A						

26. CDOG REFERENCE

a. Paragraph No. (36-44)

b. Functional Group (45)

36	37	38	39	40	41	42	43	44	45
1	4	1	2		a				6

27. FUNDING

a. Est. Total Cost (11-15)

b. % Spent Intern. (16-18)

" " Extern. (19-21)

c. Total Obligation (22-26)

d. Program. Cur. FY (27-33)

e. " " " +1 (34-40)

f. " " " +2 (41-47)

g. " " " +3 (48-54)

h. " " " +4 (55-61)

i. " " " +5 (62-68)

j. " " " +6 (69-75)

k. Total Man Years c.

Effort

(76-78)

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
					1			2							
27	28	29	30	31	32	33	34	35	36	37	38	39	40		
41	42	43	44	45	46	47	48	49	50	51	52	53	54		
55	56	57	58	59	60	61	62	63	64	65	66	67	68		
69	70	71	72	73	74	75	76	77	78						

DA FORM 1309R
1 June 63

Previous Editions are Obsolete

Page 2 of _____

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

- (A) Falkow, S., Ph.D., Dept of Bacterial Immunology
DCD&I, WRAIR, WRAMC, Washington, D.C. 20012
576-3745 or Interdepartmental Code 198, Ext 3745 49
- (A) Wohlhieter, J., Ph.D., Dept. of Bacterial Immunology
DCD&I, WRAIR, WRAMC, Washington, D.C. 20012
576-3745 or Interdepartmental Code 198, Ext 3745 49
- (A) Johnson, E.M., Ph.D., Dept of Bacterial Immunology
DCD&I, WRAIR, WRAMC, Washington, D.C. 20012
576-3745 or Interdepartmental Code 198, Ext 3745 49
- (A) Ryman, I., Dept of Bacterial Immunology
DCD&I, WRAIR, WRAMC, Washington, D.C. 20012
576-2230 or Interdepartmental Code 198, Ext 2230 49

REPORTS. Annual Progress Report, Walter Reed Army Institute of Research, 1 July 1963 - 30 June 1964.

Falkow, S., Schneider, H., Baron, L.S., and Formal, S.B.
Virulence of Escherichia-Shigella Genetic Hybrids For The
Guinea Pig. J. Bacteriol. 86:1251-1258 (1963).

Johnson, E.M., Falkow, S., and Baron, L.S. Recipient Ability
of Salmonella Typhosa in Genetic Crosses With Escherichia Coli.
J. Bacteriol. 87:54-60 (1963).

Falkow, S., Wohlhieter, J.A., Citarella, R.V., and Baron, L.S.
Transfer of Episomic Elements To Proteus I. Transfer of F-linked
Chromosomal Determinants. J. Bacteriol. 87:209-219 (1964).

Krishnapillai, V. and Baron, L.S. Alterations in The Mouse
Virulence of Salmonella Typhimurium By Genetic Recombination.
J. Bacteriol. 87:598-605 (1964).

ANNUAL PROGRESS REPORT

Project No. 3A012501B813

Title: Army Medical Basic
Research in Life Sciences

Task No. 02

Title: Microbiology

Subtask No. 02

Title: Microbial genetics
and taxonomy

Description: The purpose of these studies is to investigate the genetic characteristics of the metabolic and antigenic changes occurring in the enteric bacteria as a consequence of genetic recombination, episomic transfer, and transduction in order to examine the genetic basis of virulence.

Progress:

1. Studies on Episomic Elements

Since 1959 there has been an increasing number of reports of infectious multiple drug resistance in enteric bacteria. The most frequently encountered type of multiple drug resistance is for streptomycin (SM), chloramphenicol, and tetracycline (TC) although other combinations have been encountered and many strains are resistant to sulfonamides (SU) and Kanamycin (KM) as well. The practical significance of infectious multiple drug resistance is shown by the recent report that up to 20% of the enteropathogenic Escherichia coli serotypes isolated in Western Germany harbor infectious 5 drug resistance.

The drug resistance markers are carried and transferred by an agent, RTF, which possesses many of the properties of an episomic element. The composite agent (R-factor) composed of RTF and the drug resistance markers may be transmitted among enteric bacteria by conjugation independently of the transfer of chromosomal determinants. R-factors apparently replicate autonomously within a cell since they may be eliminated spontaneously or with acridine dyes. All available evidence indicates that R-factors behave as a single unit of transmission and replication.

Previous studies from this laboratory have clearly demonstrated that the intergeneric transfer of episomic elements between organisms which differ significantly in the overall guanine and cytosine (GC) content of their deoxyribonucleic acid (DNA) represents a unique method

of biological fractionation. During the past year we have studied the transfer of a number of R-factors from E. coli and Shigella sp. to Proteus mirabilis. DNA extracted from Proteus cells infected with R-factors exhibit a striking molecular heterogeneity in CsCl density gradients. Additional or satellite bands of DNA are recognizable in episomally infected Proteus but not in uninfected cells. Since the satellite bands may be correlated with the gain or loss of R-factors, it is felt that the satellite material represents the transferred genetic material.

Table 1 shows the genetic constitution of the R-factors studied, their source, and the mean GC composition of the DNA species which may be recognized upon fractionation of R-factor DNA. In addition, the relative size of each R-factor is listed as compared to the classical factor 222 (SU, SM, CM, TC) which comprises about 6-8% of the total DNA extracted from infected Proteus cells. The spontaneous loss of a drug marker by mutation is always associated with a decrease in the size of an R-factor which physically confirms the genetic evidence that drug marker loss is a deletion phenomenon. Moreover, loss of one or more drug resistance markers results in a change (often drastic) of the molecular distribution of R-factor DNA. For example, R-factor 222 (SU, SM, CM, TC) exhibits 3 main classes of DNA molecules with overall GC compositions of 56%, 51% and 48%, present in the proportions .35, .47 and .18 respectively. A spontaneous variant of 222, 222r₃, which has lost the TC drug marker (SU, SM, CM) although still exhibiting the same main 3 classes of molecules now has the proportions .47, .39 and .14. In other words, 222r₃ DNA shows a relative increase of .12 for the 56% GC molecules while relatively the 51% and 49% components have decreased by .08 and .04 respectively. This observation indicates that the TC drug resistant marker is composed of GC molecules with about equimolar base composition. In addition, the observed .12 relative redistribution of DNA correlates well with the .10 size reduction of the 222 to 222r₃ mutation.

These types of observations have led to the following conclusions. The TC drug marker is composed of low (50%) GC molecules while the CM drug marker is composed of high (56%) GC molecules. Together these two markers represent about 30% of R-factor DNA. The SU and SM drug markers as well as the RTF component have mean base compositions in the range 48-53%. Preliminary evidence indicates that the drug markers occupy the higher values while RTF tends towards the lower value. The KM drug marker probably has a GC composition very close to 53%. It is significant that R-factors isolated from so many widespread locations: USA, England, Germany and Japan exhibit remarkable similarity in terms of molecular makeup and size. This indicates that R-factors all belong to a single family of genetic determinants.

Table 1

Source	R-Factor	Genetic Constitution	Relative Size	Molecular species in % GC
Japan	222	SU,SM,CM,TC	1	56, 51, 49
Japan	222r ₃	SU,SM,CM	.9	56, 51, 49
Japan	N-3	SU,SM,TC	.82	53, 50, 48
Japan	R-15	SU, SM	.71	52, 50, 48
USA	RM	SU,SM,TC	.82	54, 51, 49
England	Sr ₃	SU,SM,TC	.88	53, 50, 48
Germany	r ₅	SU,SM,CM,TC,KM	1.06	56, 53, 51, 48

2. Episomic Elements in Proteus

Several Proteus strains were isolated from nature possessing the unusual ability to ferment lactose (lac^+). It was found that one of these strains, a Proteus mirabilis, could transfer the lac^+ character at high frequency to species as different as Escherichia coli, Serratia, Salmonella typhosa and Shigella, all the strains that received the lac^+ character could in turn transfer it at high frequency to other strains. The recombinants received no other genetic characters. Thus the lac^+ marker was capable of being transferred as a unit independent of the bacterial chromosome and had other properties in common with the episomic element F-lac. This genetic element detected in Proteus shall be considered an episome and termed P-lac because it resembles, but is not identical to the F-lac episome.

Ordinarily, bacterial DNA examined in a CsCl density gradient forms a unimodal distribution about a characteristic mean density which is related to the chemical composition of the DNA. However, DNA from this Proteus strain with the P-lac episome, had in addition to the characteristic Proteus DNA, a large satellite band of DNA with a markedly different base composition. The buoyant density of the Proteus DNA is 1.698 gm/cm^3 which corresponds to an average guanine and cytosine (G-C) content of 39%, characteristic of Proteus DNA. There is a satellite band of density 1.710 gm/cm^3 which corresponds to 50% G-C. This satellite is not an artifact of preparation because exactly the same banding pattern is obtained when different extraction procedures are used to isolate the DNA. The satellite DNA is always present in Proteus strains with the episome, but never present in strains without the episome.

In strain of Salmonella, Shigella, and E. coli the DNA associated with the episome has the same buoyant density as that of the normal bacterial DNA, so that the episome associated DNA does not form a satellite band. These strains still retain the ability to transfer this element and it appears again as a satellite band when the episome is transferred to a lac^+ Proteus. It is possible to measure the amount of DNA in the satellite band and it was calculated that 10% of the total DNA extracted from Proteus with the P-lac episome is in the satellite band. It is possible to purify this episomal DNA by absorption and elution from a methylated albumin keiselgar column. This purified DNA has not been shown to be biologically active because transformation is not possible with Proteus or the other enteric strains and it has yet to be tried on strains that can be transformed by DNA.

Proteus strains normally do not produce the enzymes necessary for the utilization of lactose so the amount and the nature of the enzyme produced after episome infection was investigated. The Proteus strain with the episomes produces less B-galactosidase than the coli strains with or without the episomes. Maximum enzyme activity of the cells was measured after induction for 24 hours in the presence of the substrate lactose or a synthetic galactoside.

Strain	Non-Induced	Induced
<u>Proteus</u> P-lac	12 EU/u gm. cell N	33 EU/u gm. cell N
<u>E. coli</u> W2586	0.3	109
<u>E. coli</u> P-lac	0.2	332

The regulatory mechanisms do not seem to function properly in Proteus because there is a substantial level of enzyme produced even in the uninduced cells. Coli cells with the P-lac episome produce about 3 times as much B-galactosidase as a haploid cell. This has been interpreted as an indication that there are a number of copies of the lac genes per cell.

The enzyme itself was examined to test whether the B-galactosidase produced by Proteus with P-lac was identical with the enzyme produced by E. coli K-12. Crude extracts were prepared by sonication of Proteus cells and coli cells. The sedimentation coef. and Michael's coef. were the same for both enzyme preparations. An immunological titration of the two enzyme extracts with coli B-galactosidase antiserum showed that the immunological character of the two enzymes is different. At equivalence the same amount of antiserum precipitated 42% of the coli B-galactosidase activity and only 11% of the Proteus B-galactosidase activity. A marked difference was also observed in the heat sensitivity; B galactosidase from Proteus is inactivated much faster than the enzyme from E. coli. These results are summarized in the following table.

	<u>Proteus</u> - P-lac	<u>Coli</u> - K-12
K_m	$2.06 \times 10^{-4} \text{ M}$	$1.96 \times 10^{-4} \text{ M}$
act. ppt./ml. antiserum	15.3 s	14.4 s
sed. coef.	11%	42%
at equivalence		
K_{59}	0.50 min.^{-1}	0.12 min.^{-1}

3. Chromosome Transfer Kinetics of Salmonella Hfr Strains

The mechanical interruption of chromosome transfer at regular intervals during the mating process has made possible the precise localization of genetic determinants, in units of time, in Hfr strains of Escherichia coli K-12. Recombination frequencies and linkage data obtained in this and other laboratories have indicated that Salmonella Hfr strains possess a gene order identical to K-12, and transfer their chromosomal genes to Salmonella recipients in a manner analogous to that of K-12 Hfrs. However, localization of Salmonella Hfr markers in time units by interruption of mating has not been reported. We therefore examined the kinetics of chromosome transfer of an S. typhosa Hfr and an S. typhimurium Hfr in interrupted matings with multiply auxotrophic S. typhimurium recipients.

The S. typhosa donor, labelled TD-7, was derived from S. typhosa strain 643 by mating with the S. typhimurium Hfr strain SC-19, and selecting those hybrids receiving the terminal lactose utilization (lac⁺) gene of SC-19. Since this gene is closely linked to the sex-factor, F, in SC-19, F is received along with lac⁺ by a certain percentage of the 643 hybrids, which thereby become Hfrs. SC-19 was itself derived from S. typhimurium LT-7 by a similar hybridization with the E. coli Hfr P4X-6. Thus, TD-7 has the same transfer orientation as SC-19 and P4X-6, i.e., proline synthesis (pro⁺) is injected as a lead marker and lac⁺ is the terminal genetic marker linked to F.

Interrupted matings were performed with TD-7 and the multiply auxotrophic S. typhimurium recipient strains O8R-1S^r and 74R-1S^r. The selected markers pro⁺, met⁺ (methionine synthesis), arg⁺ (arginine synthesis), and ile⁺ (isoleucine synthesis) were found to enter the recipients at 8, 32, 36, and 51 minutes, respectively, after initial contact. The order of entry of these markers is identical to that exhibited by the analogous E. coli Hfr P4X-6, confirming the identity of gene order. However, the entry times themselves were considerably different from those obtained in P4X-6 X E. coli F⁻ interrupted matings where the same markers entered at 5, 20, 22.5, and 28 minutes respectively. A similar extension of entry times was observed with S. typhimurium Hfr SR-305. SR-305 was isolated after F-infection of strain LT-2, and transfers its chromosomal markers to O8R-1SR in the reverse order ile⁺-met⁺-pro⁺ at 4 minutes, 18 minutes, and 46 minutes, respectively.

A comparison of the TD-7 entry times with those of P4X-6 (Table II) shows that for the pro, met, and arg markers the entry time ratio, P4X-6/TD-7, is constant at 0.63. This is interpreted as indicating an identical length of chromosome in the two organisms, and a rate of chromosome transfer in the Salmonella donor which is only 0.63 that of the E. coli Hfr. The possibility that the 0.55 ratio for the ile marker might indicate a longer physical length of Salmonella chromosome between met and ile is contradicted by the SR-305 kinetics, where the 14-minute interval observed between ile and met is about what would be predicted by the 0.63 ratio.

These findings suggest that the sex factor of K-12, when integrated in a Salmonella, is unable to mobilize the chromosome of that organism with the same degree of efficiency that it exhibits in mobilizing the E. coli chromosome, under the same experimental conditions.

Table II

Comparative entry times of P4X-6 and TD-7*

	<u>pro</u>	<u>met</u>	<u>arg</u>	<u>ile</u>
P4X-6	5	20	22.5	28
TD-7	8	32	36	51
RATIO (P4X-6/TD-7)	0.63	0.63	0.63	0.55

*The entry times, in minutes, for TD-7 were determined by interrupted matings with S. typhimurium strains 08R-15^R and 74R-15^R. P4X-6 entry times were determined by interrupted matings with F⁻ strains of E. coli K-12.

4. Genetics of Virulence in Salmonella

Recently, genetic systems in Salmonella typhimurium have been developed which make it possible to study extensive regions of the bacterial chromosome by recombination. The laboratory mouse is very susceptible to infection with S. typhimurium when the bacteria are introduced into the peritoneum. Many strains of the same species or related Salmonella are completely avirulent or possess intermediate levels of virulence. Since this characteristic is usually stable for any particular species of Salmonella, it was expected that a genetic basis for mouse virulence might be found.

The primary aim of this study was to determine whether the mouse virulence of the highly virulent strains of S. typhimurium could be modified by recombination with an avirulent S. abony strain. The donor strain of S. abony is completely avirulent for the mouse, being unable to multiply or produce infections in this experimental animal. It is lethal for mice only when a sufficiently high dose (about 10⁸ bacteria) is injected to produce toxicity. The C5 strains of S. typhimurium, on the other hand, are highly virulent as seen by the mortality in mice injected with even very small doses of bacteria.

Hybrids from the cross S. abony avirulent donor X S. typhimurium C5 virulent recipient were isolated and subjected to genetic analysis. Hybrids which received the his⁺, his⁺ fla⁺, or his⁺, rha⁺ markers from S. abony were unchanged in their virulence. The transfer of either the

his⁺ str-r rha⁺ region or the inos⁺ marker by itself, however, produced partially avirulent hybrids. The region involved in the determination of this difference in virulence lies within the chromosomal segment his - str - inos. Further classes of hybrids were isolated and tested and only those hybrids which possess either the str or the inos loci exhibit a decrease in their virulence. The hybrids which displayed a loss in virulence, however, did not possess the completely avirulent character of the S. abony donor.

Additional crosses were performed to determine whether an initial, partially avirulent hybrid would become completely avirulent were it to further receive the other region involved in the transition of virulence to partial avirulence. For these studies, the hybrid C5 his⁺ str-r possessing an LD₅₀ of 10⁵ bacteria was back-crossed with the S. abony Hfr, with selection being made for the rha⁺ marker of the donor. Among the classes of hybrids obtained from this backcross, only the hybrid which received the inos region, in addition to the previously transferred str region, becomes completely avirulent.

Summary and Conclusions:

1. The R-factor is an episomic element consisting of DNA which likely exists as a single molecule with heterogeneity along its length. A correlation exists between the genetic constitution of R-factors and the molecular banding pattern of DNA in CsCl density gradients. The TC drug resistant marker has an average GC composition of 50%, while the CM marker is 56%. The RTF component and the SU, SM, Kan markers have mean base compositions in the range 48 - 53% GC.

2. A strain of Proteus mirabilis was investigated which possessed the unusual ability to ferment lactose. This genetic character is transmitted by an episomic element. DNA extracted from this Proteus strain was examined by CsCl density gradient centrifugation and contained a large satellite band. The regulation of the synthesis of the inducible enzyme B-galactosidase in Proteus with the episome indicate that the repression mechanism does not function properly. The evidence also indicates that the B-galactosidase produced by the cells with this episome is different than the B-galactosidase produced by Escherichia coli K-12.

3. A Salmonella typhosa Hfr, TD-7, examined in interrupted matings with Salmonella typhimurium recipients, was found to transfer the pro, met, arg, and ile markers at 8, 32, 36, and 51 minutes, respectively, after contact with the recipient. The analogous Escherichia coli Hfr, P4X-6, transfers these markers to E. coli F⁻ strains in the same order, but at times of 5, 20, 22.5, and 28 minutes, respectively. Extended entry times were noted also with S. typhimurium Hfr SR-305, which transfers in the reverse order ile - met - pro, at 4, 18, and 46 minutes respectively. The P4X-6/Salmonella entry time ratio is constant at 0.63 for the earlier markers transferred by both TD-7 and SR-305. This suggests that these Salmonella donors transfer their chromosomes to

Salmonella recipients at a rate which is only 0.63 that of the P4X-6 transfer to E. coli F⁻ strains.

4. The results of this study demonstrate that the acquisition of partial avirulence is always associated with either the str or inos marker. The complete avirulence of the backcross hybrid which encompasses the regions of both the str and inos markers is interpreted as being due to the fact that it received both the determinants of avirulence (avir, linked to str-r and avir₂ linked to inos). Each determinant by itself would allow the phenotypic expression of partial avirulence, but both are presumably necessary for the expression of complete avirulence.

ARMY RESEARCH TASK REPORT				REPORTS CONTROL SYMBOL CSCRD-6(R2)	
ACCESSION NUMBER 16167			PROJECT, TASK, OR SUBTASK NO. DA 12591 R11020		
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D. C., 20315			2. FUNDING AGENCY Army Medical R&D Command Office of The Surgeon General Washington, D. C., 20315		
3. CONTRACTING AGENCY NA			4. CONTRACTOR AND/OR GOV'T LABORATORY Walter Reed Army Institute of Research Walter Reed Army Medical Center Washington, D. C. 20315 (202) 462-1000 Ext 2552		
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Einheber, A. Ph.D. Dept of Surgery & Burn Div of Basic Surgical Research WRAIR, WRAMC, Washington, D. C., 20315 576-3483 or Intercom Internal Code 148 Ext 2221 See Continuation Sheet 49					
6. TITLE OF: PROJECT <input type="checkbox"/> TASK <input type="checkbox"/> SUBTASK <input checked="" type="checkbox"/> Role of bacteria in shock (U)					
7. DATE OF REPORT DAY 30 MONTH 11 YEAR 1964					
8. RESUME (U) Multidisciplinary studies have been conducted on firmness and conventional animals to evaluate the pathogenesis and development of various injuries (burns, fractures, trauma, etc.) including radiation, hypothermia, hemorrhage, and shock states which are fatal if untreated or severely remediable; to establish pathogenesis; to develop methods for prevention or other means for modifying "shock response" before or after injury, and to determine the mechanisms of shock response. Therapy and histopathologic changes of shock states and their permanent occlusion of the microcirculation, and their effect on systemic vessels, circulation and "shock response" are being studied. Use and the effect of "shock response" on the microcirculation and microcirculation states are being studied. Studies are being conducted on burns by wild and domestic animals. Studies are being conducted on thyroidectomy and the effect of thyroidectomy on the microcirculation (13-thymidine) postoperative shock and the effect of thyroidectomy on systemic hypotension by using a shock model in which the shock is induced by					
9. KEY WORDS Shock, bacteria, hemorrhage, hypothermia, trauma					
10. SUPPORTING PROJECTS Not Applicable					
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet			12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet		
DA FORM 1309R 1 June 63 PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of 1					

ACCESSION NO.
36167

ARMY RESEARCH TASK REPORT

13. PROJECT, TASK OR SUBTASK NUMBER

11 22 23 24 25 26 27 28 29
3 A 0 1 2 5 0 1 B 8 1 3 0 2 0 3 1 2 6 3

14. DATE OF REPORT (30-33)

30 33 34
0 6 6 4 4

15. SECURITY OF WORK (34)

16. TYPE OF REPORT

35 36 47 48 49 50 51 52 55
3 1 2 6 3

17. SCIENTIFIC FIELD

a. Topical Classific. (56-61)
b. Functional Class (62-64)

56 61 62 64
0 1 1 0 0 0 1 1

18. OSD CLASSIFICATION

(65-66)

19. R&D CATEGORY (67)

65 66 67
B R 1

20. CONTRACT NUMBER

11 12 13 14 15 17 18 21 22 26 27
D A 1 1 1 1 1 1 1 1 1 1

21. GRANT NUMBER

28 29 30 33 34 35 36 38 39 40 41 45 46
D A 1 1 1 1 1 1 1 1 1 1 1 1

22. ESTIMATED COMPLET. DATES

47 51 52 56 57 61 62 66 67 71
1 C O N T 2 3 4 5

23. PRIORITY (11-14)

24. PROGRAM ELEMENT (15-26)

11 14 15 26
1 2 6 1 1 2 5 0 1 1

25. CMR&D CODES

27 29 30 32 33 35
N / A 1 1 1 1

26. CDOG REFERENCE

a. Paragraph No. (36-44)

b. Functional Group (45)

36 39 40 41 42 43 44 45
1 4 1 2 1 a 6

27. FUNDING

a. Est. Total Cost (11-15)

b. % Spent Intern. (16-18)

" " Extern. (19-21)

c. Total Obligation (22-26)

d. Progrmd. Cur. FY (27-33)

e. " " " +1 (34-40)

f. " " " +2 (41-47)

g. " " " +3 (48-54)

h. " " " +4 (55-61)

i. " " " +5 (62-68)

j. " " " +6 (69-75)

k. Total Man Years of
Effort (76-78)

11 15 16 18 19 21 22 26
1 1 2 1 2 1 2 1
27 28 29 33 34 35 36 40
1 1 1 1 1 1 1 1
41 42 43 47 48 49 50 54
1 1 1 1 1 1 1 1
55 56 57 61 62 63 64 68
1 1 1 1 1 1 1 1
69 70 71 75 76 78
1 1 1 1 1 1 1 1

DA FORM 1309R
1 June 63

Previous Editions are Obsolete

Page 2 of _____

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

- (A) Carter, D., Capt, MC, Dept of Germfree Research, Div of
Basic Surg Rsch, WRAIR, WRAMC, Washington, D. C., 20012
576-3483 or Interdepartmental Code 198, Ext 3483 49
- (A) Wren, R. E., B. A., Dept of Germfree Research, Div of
Basic Surg Rsch, WRAIR, WRAMC, Washington, D. C., 20012
576-3483 or Interdepartmental Code 198, Ext 3483 49
- (A) Hysell, D. K., 1st Lt, VC, Dept of Germfree Research, Div of
Basic Surg Rsch, WRAIR, WRAMC, Washington, D. C., 20012
576-3483 or Interdepartmental Code 198, Ext 3483 49
- (A) Bauer, H., M.D., Dept of Germfree Research, Div of
Basic Surg Rsch, WRAIR, WRAMC, Washington, D. C., 20012
576-3483 or Interdepartmental Code 198, Ext 3483
(Grant - Georgetown Univ School of Medicine) 49
- (A) Burnis, W., M.D., Dept of Germfree Research, Div of
Basic Surg Rsch, WRAIR, WRAMC, Washington, D. C., 20012
(Grant - Georgetown Univ School of Medicine) 49

REPORTS. Annual Progress Report, Walter Reed Army Institute of
Research, 1 July 1963 - 30 June 1964.

Levenson, S.M., Nagler, A.L. and Einheber, A.: Some Metabolic
Consequences of Shock. International Anesthesiology Clinics,
"Shock", Feb 1964, Vol 2, No. 2, Published by Little, Brown
and Co., Boston. p.237-250.

ANNUAL PROGRESS REPORT

Project No. 3A012501B813 Title: ARMY MEDICAL BASIC RESEARCH IN
LIFE SCIENCES

Task No. 02 Title: Microbiology

Subtask No. 03 Title: Role of bacteria in shock

Description:

The Department of Germfree Research, through the use of its unique technology, attempts to evaluate the manner in which bacteria and their products may modify the response of the organism to injury from physical, chemical, and viable noxae and to combinations thereof.

Study phases are in progress:

- 1) To establish and use experimental procedures which create conditions that are of interest to and of practical importance for military medicine.
- 2) To determine the local and/or systemic changes which occur, their mechanisms, and their significance to health and disease.
- 3) To compare the responses of germfree, defined-flora, and conventional(ized) animals challenged with physical, chemical, and viable noxae, or to combinations thereof; to learn the role of the microorganisms (and their products) of the internal and external environment; and the effect of their modification and/or control.

Progress:

Bowel Shock: Superior Mesenteric Artery Occlusion (SMAO)

Reduced perfusion of the splanchnic vascular bed occurs in clinical conditions which result in systemic hypotension. It has been suggested that reduced splanchnic blood flow triggers a chain of events which perpetuate the hypotension. We sought to prepare an experimental model which would allow us to observe ischemia of the bowel in the intact unanesthetized animal, thus isolating this from other factors which might be a contributing stressor to the animal.

Standardization of procedures - A metallic snare was devised with the help of Dr. I. Levin, Instrumentation Division, WRALR. The snare device is a metallic cylinder with a metal foot plate to which is attached a

length of polyethylene tubing. When the tubing is looped around an artery, it can be passed into the cylinder. A piece of metal tubing can then be crimped onto the free end of the polyethylene tubing enabling the operator to occlude and release the artery at will. The snare device offers several advantages: it can be reused; is easily sterilized; and it is impervious to attempts by the animal to remove it.

The snare device was surgically implanted into male Wistar strain rats, anesthetized with Nembutal, under aseptic conditions in the operating suite. An incision was made in the left flank and retro-peritoneal dissection was continued until the superior mesenteric artery was exposed. The loop of the snare was passed around the artery and the length of the loop adjusted. The metal tubing was crimped onto the polyethylene. The foot plate of the snare was sutured into the flank muscles in effecting their closure. The skin was then sutured closed. The rats were then returned to their cages. In one week, the rats had regained or surpassed their operative weight and appeared healthy. At this time, the snare was closed occluding the superior mesenteric artery without general anesthesia. The occlusion can be of any time interval desired and can be repeated at desired intervals. Snares have been in place for six months and are still operable.

Initially, snares were implanted in order to determine the effects of varying periods of intestinal ischemia.

Mortality and Survival Time After Temporary or Permanent SMAO

SMAO, min.	No. Rats	Mortality	Survival Time, Hrs*
45	9	0%	-
60	10	20%	15
75	10	40%	5
90	7	57%	10
105	7	71%	8
120	7	57%	10
180	8	75%	8
240	9	88%	5
Permanent	11	100%	8

* After SMAO in those dying.

Rats subjected to 120 minutes or less of SMAO showed no ill effects during the period of occlusion. After release of the artery, the majority became weak and showed bloody diarrhea. Some of these animals died and some survived. Some subjected to 180 minutes or more of SMAO began to show listlessness and diarrhea during the period of occlusion, which increased after release of the artery.

It will be noted that there was essentially no difference in survival time between animals in which SMAO was permanent and those which died from temporary SMAO of more than one hour.

Autopsies were performed. All of these animals showed areas of darkened small intestine and the lumen was filled with bloody material. The length of the dark bowel varied from the entire small bowel and cecum with bloody peritoneal fluid to a small area of approximately 3 cm. of small bowel. The area involved most constantly was the terminal ileum. (Parenthetically, the gross picture was indistinguishable from that seen in rats dying from irreversible hemorrhagic shock.) Length of survival could not be correlated with the extent or severity of hemorrhagic bowel.

Several animals were anesthetized and the appearance of the bowel was observed and photographed before, during and after SMAO. The blood in the mesenteric vessels turned dark immediately after SMAO. Approximately 90 minutes after SMAO, the bowel progressively darkened in severity and extent with time. Upon release, the blood in the mesenteric vessels rapidly became pink and the darkened bowel began to return to its normal color. The animals observed died before the bowel had returned to its normal color.

Several animals which survived a period of temporary SMAO were sacrificed after 24 and 48 hours and were found to have grossly normal bowel. The tissues were preserved in 10% buffered formalin and were subsequently examined histologically. In all cases, the aorta and superior mesenteric artery were found to be patent and no harm to these vessels was evident from the snare device.

Animals which died from temporary SMAO of three hours or less showed no lesions in the bowel. Four hours of temporary SMAO and permanent SMAO produced intestinal infarction.

One of the advantages of the snare device is that it can be used to subject animals to repeated bouts of temporary SMAO. Survivors of the primary challenge were subjected to repeated periods of temporary SMAO at 3-4 day intervals.

The following results were obtained:

First SMAO, min.	Mortality, %										
	Subsequent SMAO, minutes										
	45	60	75	90	105	120	135	150	165	180	240
45	0*	17	0	100	-	-	-	-	-	-	-
60		20*	12	0	0	0	75	100	-	-	-
75			40*	0	20	0	0	25	0	0	-
90				57*	0	0	100	-	-	-	-
105					71*	50	0	0	0	100	-
120						57*	50	0	0	100	-
180										75*	-
240											88*

* Initial mortality.

Clinically, animals subjected to repeated occlusions reacted as did those subjected to a single SMAO.

Gross examination of animals dying from repeated SMAO's showed the same picture as animals dying after a single SMAO. Histologically, however, these animals showed ulcerations in the mucosa of the small intestine with marked surrounding inflammation. No such lesions were found in animals dying from a single period of SMAO.

To summarize, an easily sterilizable, reusable snare device which can be chronically implanted in the rat and which resists efforts of the animal to remove it, was devised. It has been observed to function for periods of at least six months. This device provides a method of producing bowel ischemia of a temporary nature on repeated occasions in the intact unanesthetized animal. Periods of temporary SMAO up to three hours produced bloody bowel of varying extent and degree. The gross picture

could not be correlated with survival time. No infarctions were seen histologically. Duration of SMAO could not be used to predict mortality or survival time on an individual animal basis. Temporary SMAO for four hours or permanent SMAO produced bloody bowel of similar extent and gross appearance. Infarction could be detected histologically. Temporary SMAO resulted in no difference in survival time from permanent SMAO although mortality rate was less. There is individual variation among rats in their ability to withstand intestinal ischemia. No tolerance (ability to survive a highly lethal injury) to intestinal ischemia was demonstrated by the training regimen we followed but this does not exclude the possibility that another training regimen might not succeed. Repeated brief SMAO's, in fact, produced ulceration of the small intestinal mucosa with severe surrounding inflammation but without perforations.

Pathogenesis and treatment - No physiological measurements were made in the SMAO animals in order to add as little stress as possible to the injury inflicted. The mechanism by which SMAO causes death is not clear. Marston (Ann. Surg. 158:952, 1963), working with permanent SMAO in dogs, suggested that plasma and whole blood loss was of great significance in the pathogenesis of this injury and that blood is lost into the splanchnic bed by bleeding through the portal vein.

To estimate fluid lost into the small bowel, the weight of the small bowel, including contents, from pylorus to ileocecal valve, was compared between normal animals and those exhibiting the most severe hemorrhagic bowel after fatal temporary SMAO. The average figures were:

Normal Bowel (8 rats)	SMAO Bloody Bowel (6 rats)
4.5 Gm.	6.8 Gm.

An increase in weight, presumably due to fluid loss, was noted. We, therefore, sought to determine whether parenteral fluid administered to the rats would increase either the survival rate or survival time.

Male rats with snares implanted as described above were allowed to recover from surgery for 7 days. They were divided into two paired groups by weight. Both groups were subjected to 180 minutes of SMAO. The treated group received 30% of their body weight in saline just prior to release of the SMAO. This treatment has been found to be highly effective in protecting mice from limb ischemia shock that is ordinarily fatal.

The following results were obtained and showed no statistically significant differences:

	No. Rats	Mortality	Survival Time
Untreated	6	83%	3.5 Hrs.
Saline-Treated	7	71%	9.7 Hrs.

A second group of animals with snares implanted were divided into two equal groups. The control group had 3 hours of SMAO and were then anesthetized with ether, and the external jugular vein isolated. The wound was closed, the occlusion released, and the animals were returned to their cages. The treated group was treated similarly, but the external jugular vein was cannulated, 5 cc. of Low Molecular Weight Dextran (average molecular weight 35,000) was given i.v., 20 cc. saline was given i.p., the occlusion released and the animals were returned to their cages after the neck wounds were closed. The following results were obtained and showed no statistically significant differences:

	No. Rats	Mortality	Survival Time
Untreated	3	100%	4.0 Hrs.
Treated	3	100%	13.3 Hrs.

To determine whether retrograde bleeding from the portal vein occurred when arterial pressure was reduced by SMAO, a series of acute experiments was carried out. Male Fischer rats were fasted the night preoperatively. The abdomen was sheared, anesthesia was induced and maintained with Fluothane. Surgery was performed under aseptic conditions in the operating suite. Various combinations of visceral vessels were permanently occluded; in all cases, arteries were occluded before veins. The laparotomies were then closed in two layers, the animals rapidly awakened from the light anesthesia and were observed until death. Autopsies were performed and tissues were preserved.

If retrograde bleeding from the portal vein into the superior mesenteric vein (SMV) and the intestinal vascular bed largely accounts for the blood loss, then, conceivably, occlusion of the SMV or the

celiac artery (CA) might curtail such blood loss and result in an increase of survival time beyond that of animals subjected to SMAO alone. Furthermore, animals subjected to SMV, CA and SMA occlusions might be expected to show pale rather than bloody bowel at autopsy. The following results were obtained, however:

Occlusion	No. Rats	Mortality	Survival Time
SMA	6	100%	9.0 Hrs.
SMA & SMV	12	100%	6.5 Hrs.
SMA & CA	12	100%	5.2 Hrs.
SMA, CA, SMV	6	100%	1.9 Hrs.

Unsurprisingly, all animals died. All showed bloody bowel. In the animals with SMA and CA occlusion, the livers were also congested and the animals convulsed prior to death. The cecum was congested in a number of these animals, but this finding was not constant. Guaiac positive material was present in the lumina of all the intestinal tracts.

Histologically, all specimens showed degrees of damage varying from moderate to severe. The severely damaged areas showed coagulation necrosis, while the less damaged areas showed minute, superficial ulcerations. The cecum showed the same picture as the moderately damaged areas of the small intestine. No correlation could be made between the histological picture observed and the vessels occluded or the length of survival.

An additional six rats subjected to permanent occlusion of the celiac artery survived past 48 hours. Postmortem examination showed congested livers and hemorrhagic stomachs. No other lesions were noted.

To summarize, the presence of grossly and histologically apparent hemorrhagic small bowel and the presence of bloody material in the intestinal lumen indicate that blood loss may be one of the factors in the lethality of SMAO. The amount of blood present in the small bowel and its contents is apparently not large when grossly hemorrhagic intestines are compared with normal intestines by weight. Parenteral fluid therapy did not significantly change survival time or mortality rate. The source of the hemorrhage into the small bowel is not clear. Occlusion of the superior mesenteric and celiac arteries, followed by

occlusion of the superior mesenteric vein resulted in small bowel hemorrhage and a survival time that was less than when SMAO alone was performed. Further studies are in progress to determine the mechanisms of lethality in SMAO in conventional animals.

SMAO in germfree and conventionalized animals - Reduction in splanchnic blood flow has been shown in nearly all forms of systemic hypotension. Hemorrhagic small bowel is present and very similar in appearance in rats dying from irreversible hemorrhagic shock and from SMAO.

Bacterial factors have been implicated by Fine and his associates in the pathogenesis of irreversible hemorrhagic shock and are presumed to enter the system circulation through the ischemic intestine. Furthermore, recent work by Amundsen and Gustaffson (J. Exp. Med. 117: 823, 1963) and Cohn et al. (Ann. Surg. 156: 692, 1962) has shown that germfree animals tolerate strangulation obstruction far better than do their conventional counterparts and has implicated bacterial factors in the lethality of strangulation obstruction.

Utilizing our experience with open-room conventionalized rats, we, therefore, sought to determine the course of SMAO in germfree animals to compare it with the course in conventionalized animals.

Male germfree Fischer rats obtained from the Charles River Breeding Laboratories were used. At six weeks of age, they were divided into two groups and one group was conventionalized with the cecal contents of open-room conventional rats. One month later, the animals were used. Two series of experiments were performed - the first a study of temporary (180 min.) SMAO and the second a study of permanent SMAO. In the first group, a snare device was implanted as described previously (surgery performed in a surgical isolator) and animals were subjected to 180 min. SMAO. Both groups of animals dying from SMAO showed hemorrhagic small bowel and cecum. The following mortality results were obtained and showed no statistically significant differences:

Microbial Status	Mortality	Survival Time
Germfree	75%	3 Hrs.
Conventional	67%	15 Hrs.

A comparable group of animals were used in another study. They were fasted the night preoperatively. The flank was depilated, and anesthesia was induced and maintained with Fluothane. Surgery was performed in a germfree surgical isolator. The SMA was tied, the wound closed, animals awakened very shortly from the light anesthesia, and they were observed until death. The following results were obtained:

Microbial Status	No. Rats	Mortality	Survival Time
Germfree	5	100%	5.5 Hrs.
Conventional	5	100%	5.5 Hrs.

At autopsy, the gross picture was the same in the two groups. The small bowel and cecum were hemorrhagic.

Histologically, some segments of the small bowel showed complete infarction in both groups. In less severely damaged areas, both groups showed superficial mucosal ulcerations attended by varying degree of inflammation and exudation into the intestinal lumen. The ulcerations and degree of inflammation were milder in the germfree than in the conventional group, however.

In summary, preliminary observations do not point up any marked differences between the course of SMAO in germfree and conventional rats. Further studies are in progress.

Limb-Ischemia (Tourniquet) Shock

On the basis of our experience and findings with tourniquet shock in open-room conventional ICR mice and past studies with tourniquet shock in defined-flora and germfree mice which suggested that bacteria play a role in this injury (Levenson, Einheber, and Malm, JAMA 181:874, 1962), we are extending these studies.

Saline-irreversible tourniquet shock - Adequate amounts of isotonic sodium salts have been found to be highly effective in promoting survival of mice after they have received "one lethal dose" of burn, tourniquet injury or hemorrhage. However, it has been reported (Rosenthal, S.M. in The Biochemical Response to Injury, C C Thomas, 1960, p. 397) that few mice survive more than one lethal dose of trauma (tourniquet to four legs) "in spite of therapy with up to 30% body weight saline even when supplemented with plasma or whole blood and antibiotics." The difficulty of explaining "why doubling the trauma is not to some extent counteracted with the double the amount of

therapy", has led Rosenthal to consider this as evidence that factors in addition to fluid, electrolyte and protein disturbances are acting in shock. If we can substantiate these findings with four leg tourniquets, then it would be of interest to repeat these experiments with germfree mice to determine whether or not a bacterial factor is involved, i.e., whether these mice are more tolerant of this injury than conventional mice.

We have been able to corroborate Rosenthal's observation by demonstrating an episode of limb-ischemia that is refractory to saline therapy. This we have done by contrasting the survival response of open-room conventional ICR mice given saline i.p. in amounts calculated to be therapeutic when administered immediately after release of isolateral tourniquets (both a front and hind limb on either the left or right side of the mouse) or of quadrilateral tourniquets (all four limbs) that were applied for 3.5 hours (Table 1). In preliminary studies, we demonstrated additionally: that no difference in mortality occurred in mice subjected to either right or left-sided isolateral tourniquet injury, and that both procedures responded equally and successfully to 15% body weight saline i.p.; and that tourniquets ("T") could be left on all four limbs for 48 hours without a fatality. In fact, removal of the tourniquets at this time did not result in death during the next 48 hours the mice were observed.

Table 1

"T" Time, Hours	No. Mice	Tourniquet Procedure	Therapy at "T"-Off, I.P.	Mortality, %		
				Hrs Post-	"T"-Off	
				6	24	48
3.5	35	Quadrilateral	None	80%	100%	100%
3.5	39	Quadrilateral	30% B.W. Saline	13%	69%	87%
3.5	50	Isolateral*	None	30%	88%	94%
3.5	50	Isolateral*	15% B.W. Saline	0%	4%	32%

*Results of right and left isolateral tourniqueted animals pooled.

While a degree of protection is provided up to 24 hours after quadrilateral tourniquet injury by 30% body weight saline, the benefit is lost by 48 hours. The isolateral tourniquet injured animals are definitely protected by 15% body weight saline therapy, and the survivors that were realimented after 48 hours proved to be indefinite survivors. We are in the process of determining the amount of fluid lost into the ischemic tissue following isolateral or quadrilateral tourniquets. If the amount lost after the latter is less than twice that lost after the former, then the refractoriness of the mice to a doubling of saline therapy from 15 to 30% of the body weight would be even a greater enigma.

Response of germfree and conventionalized mice to limb ischemia - Ten-month-old germfree and conventionalized ICR mice, of either sex, were subjected to limb-ischemia shock by placing rubber band tourniquets on all four limbs (quadrilateral tourniquets). Tourniquets were applied to the limbs of the mice for either 3 hours or 48 hours. All mice that received 48-hour quadrilateral tourniquets were untreated. Mice subjected to 3-hour quadrilateral tourniquets were either not treated or were given an i.p. injection of sterile isotonic saline amounting to 30% of their body weight immediately after tourniquet release. All mice were weighed and given identifying color markings on the day before experimentation. All mice were allowed food (L-356 diet) and water ad libitum before experimentation, but none after. Tourniquet procedures were carried out within a two-man plastic operating isolator. The animals were transferred from their holding isolators to the operating isolator for tourniquet application and then back again for observation. The technique we used to apply the tourniquets to the hind limbs of the mice is the same we have standardized and used in the open laboratory (see Ann. Prog. Rept., Role of Bacteria and Endotoxins in Shock, 1 Jul 62 - 30 Jun 63). The technique we used to apply tourniquets on the forelegs was a slight modification of our foreleg procedure used in the open laboratory. Within the isolator, a size 18 rubber band (Janus) was wrapped 12 turns around a No. 5 cork borer and one blade of a pair of blunt forceps. This forceps blade was then used as a lever to discharge the rubber band onto the mouse's foreleg. After the tourniquets were applied, all mice were wrapped in aluminum foil in an effort to prevent them from biting their legs and/or the rubber band tourniquets ("T"). They were then housed in separate compartments until the termination of the experiment. Mortality checks were made at frequent intervals and recorded. All dead mice were examined grossly and placed in 10% buffered formalin. At the end of the 48-hour observation period, all surviving mice were killed by cervical dislocation and likewise examined and fixed in formalin. The following results (Table 2) have, thus far, been obtained:

Table 2

Microbial Status	No. of Mice	"T" Time, Hrs	Therapy at "T", i.p.	Mortality, %		
				Hrs. Post-Release of 3 Hr Tourniquets or After Application of 48 Hr. Tourniquets		
				6	24	48
Germfree	3	48	None	0%	0%	0%
	14	3	None	93%	100%	100%
	22	3	30% B.W. Saline	64%	59%	64%
Conventional	3	48	None	0%	0%	0%
	6	3	None	83%	100%	100%
	14	3	30% B.W. Saline	22%	57%	71%

These pilot experiments indicate that quadrilateral tourniquets kept continuously in place for 48 hours are not lethal for either germ-free or conventionalized mice. Regardless of saline therapy, germfree and conventionalized mice show no difference in mortality. Further studies are planned with younger mice.

Burns

One of our objectives is to study the extent to which bacterial factors influence early mortality (shock phase), delayed mortality and convalescence after burns alone or when combined with ordinarily low lethal x-irradiation (Levenson, Einheber, and Crowley, Research in Burns, Publ. No. 9, AIBS, 1962, p. 143). We have been performing preliminary experiments with open-room conventional ICR mice to standardize burn equipment and procedures, anesthesia and mortality so as to enable us to study burns with germfree and defined-flora animals.

Early and delayed mortality - The following observations have been made: Open-room conventional ICR mice of either sex were subjected to a back burn scald at various temperatures (85, 83, 80 or 77°C.) for 10 seconds. This burn involved 30-40% of the body surface. Water was maintained at the desired temperature by a Bronwill water temperature regulator and circulator which we have found sterilizable with ethylene oxide. The mice were scalded in a stainless steel receptacle containing 6 liters of water to which were added 5 ml of "Tween 80". The latter served to insure uniform wetting of the unshaved dorsum of the mouse during scalding. Mice were allowed food (L-356 diet which is fed our germfree mice) and tap water ad libitum before the burn, but none for 48 hours thereafter. Mice were then anesthetized with either an i.p. injection of 40 mg/Kg body weight of Nembutal or exposed to a 3% concentration of Fluothane in air for 5 minutes and subjected to scald. An effort was made to burn the same area in each mouse. After the burn, the dorsum of the mouse was immediately immersed in room-temperature water (25°C) for 10 seconds ("cooled"), except when stated as otherwise. All animals were then carefully dried with surgical gauze pads or absorbent cellulose. At this time, mice were either not treated or were given an i.p. injection of sterile isotonic saline amounting to 15% of body weight. These mice and their untreated counterparts were then placed in individual wire-floor cages for observation. A record was maintained of the mortality and of the ambient temperature and humidity.

Effect of saline therapy with post-scald "cooling" of the burned region - All untreated mice that were burned at 85°C. for 10 seconds were dead by 24 hours. Mice given 15% body-weight isotonic saline were afforded some temporary protection during the first 24 hours following the burn of 85°C., but were not permanent survivors. Mice, both male and female, that were burned at either 83, 80 or 77°C. for 10 seconds received significant permanent benefit from the saline therapy.

Effect of saline therapy and post-scald "cooling" vs no "cooling" of the burned region - Forty-five mice were burned at either 80 or 77°C. for 10 seconds. Some of these mice were "cooled" after the burn, the others were not. All mice were given saline therapy after they were dried. These preliminary results suggest mortality is greater for mice that are not "cooled" after the burn than for those that are.

Sex factors and post-burn mortality - We have compared the mortality, both acute and delayed, of male and female ICR mice that were given the back burn injury at 80°C. for 10 seconds. Approximately half the males and females were treated with saline, the other half were untreated. The data, thus far, suggest that the untreated female is more resistant during the shock period than the untreated male. However, both respond comparably to saline therapy. By contrast, the impression gained from the above data is that the saline-treated female mice may be more susceptible to delayed death (7-14 days post-injury) than are similarly burned and treated males of the same age. More observations are required to be certain of these sex differences.

Effect of Nembutal vs Fluothane anesthesia preceding burn injury - The data presently indicate no difference in mortality response of the mouse to burn injury (treated with saline or not) whether prescald anesthesia is 40 mg/Kg of Nembutal or 5 minutes inhalation of a 3% mixture of Fluothane and air. It has been our experience, however, that chloroform anesthesia does adversely effect the survival of burned mice.

Pathological and microbiological findings in burned open-room conventional mice - Immediately following the burn, the skin appeared pale and ischemic. Some animals that were sacrificed approximately 1 hour after being burned displayed a hyperemic skin. One week post-burn, the burned skin began to separate from the underlying tissue at its margins. This sloughing of the eschar progressed until the entire area of the burn was a mass of exposed granulating tissue. Healing and scarring-in did not occur completely until several months had elapsed. By careful observation of the animals and gross examination at the time of sacrifice, we were able to pick up any upward responses to the burn injury. Pseudomonas aeruginosa was isolated from one animal which still appeared sick two months after the burn. This animal had numerous fistulous tracts in the area of the burn as well as a purulent peritonitis and splenic abscess. This strain of Pseudomonas is planned to be used in future gnotobiotic mouse burn studies.

In summary, it appears that there is a maximum burn time and temperature at which an i.p. injection of physiological saline, amounting to 15% of the mouse's body weight is effective in preventing fatal shock. With more severe burns, this dose of saline appears to have no effect in reducing or delaying death in the shock period (0-48 hrs. post-burn). However, with suitable burn conditions, 15% b.w. isotonic saline is highly efficacious shock therapy. Once the animals survive the

Microbial Status	No. of Mice	Mortality, Hrs Post-Burn*		
		6	24	48
Germfree	19	74%	89%	100%
Conventional	18	67%	83%	100%

* Since there was no difference between saline treated and non-treated burned mice, the data have been combined. Burn temperature 80°C. for 10 seconds.

In any event, the burn was too severe to demonstrate any difference in response to saline therapy, and therefore, possibly, to the microbial status. Experiments are in progress in which: younger mice will be used; a less severe burn will be inflicted followed by immediate immersion of the burned region into 25°C. water; and Fluothane instead of pentobarbital sodium anesthesia will be used.

Induction of Local Protection Against Burn Injury - R. Hoene (Arch. Path. 58:214, 1954; PSEBM, 85:56, 1954) described precise experimental conditions under which mild heating in water (48°C. for 2.25 minutes) of the rat's hind paw resulted in a transient inflammation which was followed by resistance of this paw to a second more severe and otherwise damaging heat challenge (49°C. for 2.5 min.) applied 3 days later. The mechanism of this locally acquired resistance to burn injury is not known, nor is it clear what accounts for the associated regional lymph node responses, viz., edema and hemorrhage.

Because, under normal laboratory conditions, the burned paw of the rat is exposed to environmental bacteria which may contribute to the picture seen, we have considered studying Hoene's phenomenon in germ-free animals. The idea is to separate the response due to tissue damage per se from that arising secondarily from infection and bacterial factors.

Our preliminary gross and histopathological observations of Hoene's phenomenon on open-room conventional Fischer rats have, for the most part, confirmed his findings. In addition, we have found that environment seems to have a marked effect on healing time. Several rats' paws were burned with the severe scald (49°C. for 2.5 minutes) and the rats then divided into two groups. One group was allowed to run around in wood chip bedding while the other was separated from the bedding by a wire mesh floor. The animals on wire healed much faster. This may be due to the cleaner surroundings and lesser contamination of the burn wound by the bedding and excreta.

We also made an attempt to ascertain the length of protection of the paw to burn injury. Three months after challenging a single hind paw of rats with the severe burn (49°C. for 2.5 minutes) (when injury to these paws was no longer apparent), both hind paws were exposed to the severe scald. The previously burned paw showed only nominal swelling, in

contrast to the other, previously uninjured paw which became swollen and necrotic.

Routine histopathological observations have, thus far, not given us a clue as to the mechanism of protection. Further studies of this phenomenon are in progress.

Radiation Injury and Associated Biological Phenomena

Studies on the effect of the microbial flora on radiation response were continued in collaboration with the Division of Nuclear Medicine.

Supralethal whole body x-irradiation - In order to substantiate the longer survival time already found for germfree mice after exposure to supralethal amounts of radiation (25,000 r and 40,000 r), mice were exposed to 35,000 r. A mean survival time of 69 hours was found for germfree mice while conventional mice survived an average of 22 hours.

Age vs. radiation sensitivity - A group of old germfree mice (40 weeks) were exposed to 1800 r x-radiation. The survival time for this age group was not significantly different than that for younger mice (12-20 weeks). This study can be completed when mice of young age groups (4, 6, 8 weeks) become available.

Determination of LD₅₀ dose of whole body x-irradiation for ICR mice - The LD₅₀ curve for the strain of mice now commercially available (ICR) was completed. Germfree mice were found to have a higher (810 r) LD₅₀ than conventional mice (640 r.). This confirms the finding already reported for the ND-2 strain (no longer available to us). The germfree mouse is able to tolerate more x-radiation than its conventional counterpart.

Radio-iodine studies (Thyroid Activity) - Metabolic rate is known to influence post-irradiation mortality in conventional mice. Inasmuch as germfree and conventional mice show a difference in radiation response, the status of thyroid activity has been measured in both by use of radio-iodine. Whole body retention of I¹³¹ was found to be greater for the germfree mouse. This difference appeared within 6 hours after i.p. administration of tracer amounts of I¹³¹ and persisted throughout the 8 days of the study. In a second phase of this study, germfree and conventionalized male and female mice were given I¹³¹ and after determination of whole body retention the radio-iodine content of the thyroid gland and serum was measured. In addition the protein bound iodine fraction expressed as conversion ratio is being studied in an effort to determine if there is a difference in thyroxine production between germfree and conventional mice. Evaluation of the data has not yet been completed.

Renewal of intestinal epithelium after x-irradiation, studied with tritiated thymidine - The germfree mouse has a longer survival time after x-ray doses producing 3-5 day deaths. Since the rate of cell renewal

in intestinal epithelium is much slower in germfree mice, and since radiation damage is known to be reduced in tissues with low rates of cellular proliferation, a study has been started to determine if this difference in cell renewal is responsible for the increased survival time in germfree mice. Tritiated thymidine was injected i.p. 12 hours before exposure to 1500 r x-ray. Animals were sacrificed at 12-hour intervals post-radiation. Sections of ileum, duodenum and jejunum and radioautographs are being made from these sections. This study is not yet complete.

Trace metals by neutron activation analyses - In an effort to determine basic differences between germfree and conventional animals which may relate to differences in radiosensitivity, biochemical, radiochemical, and neutron activation analyses are planned. Serum, urine, and muscle tissues from control and irradiated rats and mice have been obtained and are being preserved until analyses can be performed. (For details, see Annual Progress Report, RD 44-61 Biomedical (NWER) (DASA), 03.074 (Biological effects of total and partial body irradiation at cellular, organ, and total organism level).

Pneumatic Suction for Induction of Hypotension

In the last year's annual progress report, Project # 3A0 12501 A 802 01, Department of Germfree Research, the usefulness of, and progress toward the development of, a mechanical device for applying controlled pneumatic suction to the hind limbs of the anesthetized, supine monkey were detailed.

Further progress was made during the past year. A mechanical device was tested and found to induce hypotension in the monkey. With the aid of Dr. Fred Leonard of the Department of Biomechanical Instrumentation, two lucite chambers were adapted to fit across the inguinal line with latex-coated stockinette shaped to exactly fit the legs of the monkey. Facilities were provided on the lucite chambers so that suction could be applied to either leg separately and the negative pressure applied could be measured.

A negative pressure of 60 mm of Hg to one leg promptly dropped the monkey's blood pressure 15-20 mm of Hg, but the blood pressure gradually returned to normal. 90-120 mm of Hg negative pressure to both legs promptly dropped the blood pressure to 20 mm of Hg. This slowly returned to normal after pressures in the lucite chambers were allowed to return to normal.

In summary, a suitable device for applying controlled pneumatic suction to the hind limbs of the anesthetized monkey was devised and tested. Hypotension of a severe nature was induced with this device.

Facilities for housing and working with large animals are no longer available to this department. Opportunity for exploiting the advantages of this device in the future will depend upon collaboration with other departments.

Adrenal Physiology: Effect of Diet on Survival Post-Adrenalectomy

Adrenalectomy renders animals more susceptible to stress. Thus, adrenalectomized mice are highly susceptible to experimentally induced infections and to administered endotoxins; the influence of steroids on the reticulo-endothelial system has been studied but is incompletely understood. As regards the therapeutic efficacy of adrenocortical steroids, both gluco- and mineralo-corticoids, in clinical and experimental infections and "endotoxinemias", the literature is confusing and conflicting. Due to the well known hypersecretion of steroids following injury and infection and other noxious stimuli, it has been suggested that to discern the "non-specific" effects of the stressors, which relate in large part to the steroids, it is instructive to study the responses to stressor agents, of adrenalectomized as well as intact animals. To our knowledge, germfree animals have not been adrenalectomized and their ensuing metabolic and histopathologic states contrasted with that of adrenalectomized conventional animals. In view of the aforementioned, and our continuing interest in traumatic shock and infection, wound healing, inflammation, endotoxins, etc., it seems important to us to determine initially the clinical course and survival of adrenalectomized germfree and conventional animals. Before studying and comparing survival and the clinical and biochemical course of germfree and conventionalized animals after adrenalectomy, we have been attempting first to standardize procedures in open-room conventional rats and mice. However, as reported previously (Annual Progress Report, 1962-63); we encountered the difficulty that after adrenalectomy, both species maintained on our standard L-356 diet and offered demineralized water instead of saline, contrary to expectation, failed to die. When diet and fluid were removed and the adrenalectomized rats allowed to die of starvation, the rats previously on demineralized water did, however, die sooner than those that had been maintained on saline. Moreover, all adrenalectomized rats died of starvation before the sham-adrenalectomized rats. Since adrenal cortical tissue was not found on post-mortem examination of the adrenalectomized animals, it appeared that our standard powdered L-356 diet, purchased from commercial sources, provided an adequate level of sodium. Because, in our experience, this is the best diet available for feeding germfree rats and mice and is the only diet we use, we purchased, on special order, L-356 diet containing the standard salt mix from which the sodium chloride had been excluded. Using this "sodium deficient" L-356 diet, we sought to determine the survival ability of adrenalectomized open-room conventional ICR mice by studying the following four groups: adrenalectomized and sham-adrenalectomized mice given saline and regular L-356 diet and adrenalectomized and sham-adrenalectomized mice given demineralized water and "sodium deficient" L-356 diet. A total of 24 mature mice were used. Each of the four groups consisted of 3 males and 3 females. Body weight was recorded daily. All mice survived a period of 24 days, at which time they were sacrificed. Gross and histologic search was made for the presence of adrenocortical tissue. All groups suffered a comparable, albeit transient weight loss over the first 2 days post-surgery, after which body weight returned to and remained at pre-operative levels in all groups. Careful examination of the carcasses revealed no accessory nodules or remaining adrenal cortical tissue.

The fact that these mice survived suggests two possible explanations: either the observation period was too short or the "sodium deficient" diet was not deficient enough, i.e., contained ample sodium to sustain life indefinitely. We can only speculate, at present, that the latter is the likely explanation because some weight loss or other manifestations would have been in evidence, by 24 days, if these mice would have died subsequently. It has become standard procedure to provide adrenalectomized animals with saline in lieu of drinking water. It would appear that the routine offering of saline may in many instances be superfluous, or perhaps even harmful, unless the adequacy of the sodium content of the feed be ruled out. In any event, we hope to be able to explain more precisely why the open-room conventional ICR mouse and Fischer rat survive adrenalectomy for extended periods in our laboratory. Meanwhile having found that intact germfree rats, as do intact germfree mice, tolerate the lethal effects of starvation more poorly than their conventionalized counterparts, we may study whether this pattern persists after adrenalectomy.

SUMMARY AND CONCLUSIONS:

Multidisciplinary studies have been conducted on germfree and conventional animals to evaluate the pathobiological evolution of various injuries (bowel-ischemia, limb-ischemia, burns, ionizing radiation, limb decompression, adrenalectomy) and the resulting shock states which are fatal if untreated or presently irremediable; to establish pharmacologic, physiologic, environmental, or other means for modifying "shock-resistance" before or after injury; and to determine the mechanisms of such modification. Therapy and histopathologic changes of shock after temporary or permanent occlusion of the superior mesenteric artery and other splanchnic vessels; standardization of burn procedure for germfree isolator use and the effect of sex, immersion-"cooling", anesthesia and microbial status on post-burn survival; local protection against burns by mild pre-irritation; effect of microbial status on thyroid activity (I^{131}) and renewal of intestinal epithelium (H^3 thymidine) postirradiation; and a device for inducing systemic hypotension by limb-decompression were studied.

ARMY RESEARCH TASK REPORT		REPORTS CONTROL SYMBOL CSCRD-6(R2)
ACCESSION NUMBER 36168		PROJECT, TASK, OR SUBTASK NO. 3A012501B8130321
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D. C., 20315	2. FUNDING AGENCY Army Medical R&D Command Office of The Surgeon General Washington, D. C., 20315	
3. CONTRACTING AGENCY NA	4. CONTRACTOR AND/OR GOV'T LABORATORY A Walter Reed Army Inst of Rsch Walter Reed Army Medical Center Washington, D. C., 20012 723-1000, Ext. 3552 49	
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Campbell, William J. Capt, MSC, Dept of Biological Chemistry, Division of Biochemistry, WRAIR, WRAMC, Washington, D. C., 20012 576-3528 or Interdepartmental Code 198, Ext. 3528 See Cont. Sheet 49		
6. TITLE OF: PROJECT <input type="checkbox"/> TASK <input type="checkbox"/> SUBTASK <input checked="" type="checkbox"/> Biochemical activity in health and disease. (U)		
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964		
8. RESUME (U) Effect of chromium(III) on mitochondrial swelling was investigated in the presence of insulin. Differences in substrate oxidation were noted. Biological activity of stressor substances has been studied and additional methods developed for assays. Studies of lipid soluble vitamins have shown responses in animals attributed to varying Vitamins A and E in experimental diets. A new method for transesterification of fatty acids eliminates interfering infrared absorption in determining trans isomers of unsaturated fatty acids, (UFA). Rats fed high concentrations of TUFA appear to be unaffected. Disc electrophoresis and thin film chromatography are being adapted to study lipoproteins and mucoproteins of serum. Organophosphorus compounds have been synthesized which have varying antitrypsin activity according to molecular structure. Improved countercurrent distribution procedures allow isolation of purified alanine and tyrosine RNA in relatively large amounts. Two serine acceptor RNAs have been separated and purified from yeast. Electroanalytical chemistry has been extended to the determination of biologically and clinically important substances. Glucose metabolism of normal & parasitized erythrocyte has been studied in malarial infections. A field survey of vitamin, trace metals, and protein nutrition was conducted in Thailand in various age groups. Results of this survey are currently being compiled.		
9. KEY WORDS Chromium, insulin, adrenaline, stress, vitamins, trypsin, proteins, mucoproteins, nucleic acids, glucose, malaria		
10. SUPPORTING PROJECTS Not applicable.		
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet		12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet
DA FORM 1309R 1 June 63		PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of ____

ACCESSION NO.

36168

ARMY RESEARCH TASK REPORT

13. PROJECT, TASK OR
SUBTASK NUMBER

11	22	23 24	25 26	27 28	29
3 A 0 1 2 5 0 1 B 8 1 3	0 3	2 1			

14. DATE OF REPORT (30-33)

30	33	34
0 6 6 4	4	

15. SECURITY OF WORK (34)

16. TYPE OF REPORT

35	36	47 48	49 50	51	52	55
3					1 2 6 3	

17. SCIENTIFIC FIELD

 a. Topical Classific. (56-61)
 b. Functional Class (62-64)

56	61	62	64
0 1 0 6 0 6			

18. OSD CLASSIFICATION

(65-66)

19. R&D CATEGORY (67)

65 66	67
B R	1

20. CONTRACT NUMBER

11 12	13 14	15	17	18	21	22	26	27
D A								

21. GRANT NUMBER

28 29	30	33	34 35	36	38	39 40	41	45	46
D A							G		

22. ESTIMATED COMPLET.
DATES

47	51	52	56	57	61	62	66	67	71
1 C O N T	2			3		4		5	

23. PRIORITY (11-14)

24. PROGRAM ELEMENT
(15-26)

11	14	15	26
	2	6 • 1 1 • 2 5 • 0 1 • 1	

25. CMR&D CODES

27	29	30	32	33	35
N / A					

26. CDOG REFERENCE

a. Paragraph No. (36-44)

b. Functional Group (45)

36	39	40	41 42	43 44	45
1 4 1 2			a		6

27. FUNDING

a. Est. Total Cost (11-15)

b. % Spent Intern. (16-18)

" " Extern. (19-21)

c. Total Obligation (22-26)

d. Progrmd. Cur. FY (27-33)

e. " " " +1 (34-40)

f. " " " +2 (41-47)

g. " " " +3 (48-54)

h. " " " +4 (55-61)

i. " " " +5 (62-68)

j. " " " +6 (69-75)

k. Total Man Years of

Effort

(76-78)

11	15	16	18	19	21	22	26
		1		2			
27 28	29	33	34 35	36	40		
41 42	43	47	48 49	50	54		
55 56	57	61	62 63	64	68		
69 70	71	75	76	78			

DA FORM
1 June 63 1309R

Previous Editions are Obsolete

Page 2 of

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

- (A) Soldevila, M. D., Capt, MSC, Dept Biol Chemistry,
Div Biochemistry, WRAIR, WRAMC, Wash. D. C. 20012
576-3528 or Interdepartmental Code 198, Ext. 3528 49
- (A) Smith, M. J., Pfc, Dept Biol Chemistry,
Div Biochemistry, WRAIR, WRAMC, Wash. D. C. 20012
576-3528 or Interdepartmental Code 198, Ext. 3528 49
- (A) Decker, W. J., Capt, MSC, Dept Biol Chemistry,
Div Biochemistry, WRAIR, WRAMC, Wash. D. C. 20012
576-3528 or Interdepartmental Code 198, Ext. 3528 49
- (A) Knoblock, E. C., Lt Col, MSC, Div Biochemistry
WRAIR, WRAMC, Washington, D. C. 20012
576-3528 or Interdepartmental Code 198, Ext. 3528 49
- (A) Mehlman, B., MS, Dept Clin Chemistry,
Div Biochemistry, WRAIR, WRAMC, Wash. D. C. 20012
576-3528 or Interdepartmental Code 198, Ext. 3528 49
- (A) Corwin, L. M., PhD, Dept Biol Chemistry,
WRAIR, WRAMC, Washington, D. C. 20012
576-3528 or Interdepartmental Code 198, Ext. 3528 49
- (A) Cohen, S. N., Capt, MC, Div Biochemistry,
WRAIR, WRAMC, Washington, D. C. 20012
576-3528 or Interdepartmental Code 198, Ext. 3528 49
- (A) Fanning, G. R., MS, Dept Biol Chemistry,
Div Biochemistry, WRAIR, WRAMC, Wash. D. C. 20012
576-3528 or Interdepartmental Code 198, Ext. 3528 49
- (A) Jordan, W., MS, Dept Clin Chemistry,
Div Biochemistry, WRAIR, WRAMC, Wash. D. C. 20012
576-3528 or Interdepartmental Code 198, Ext. 3528 49
- (A) Faulkner, A., MS, Dept Clin Chemistry,
Div Biochemistry, WRAIR, WRAMC, Wash. D. C. 20012
576-3528 or Interdepartmental Code 198, Ext. 3528 49
- (A) Boone, B. J., BS, Dept Biol Chemistry,
Div Biochemistry, WRAIR, WRAMC, Wash. D. C. 20012
576-3528 or Interdepartmental Code 198, Ext. 3528 49
- (A) Doctor, B. P., PhD, Dept Biol Chemistry,
Div Biochemistry, WRAIR, WRAMC, Wash. D. C. 20012
576-3528 or Interdepartmental Code 198, Ext. 3528 49

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

- (A) Connelly, C. M. , MS, Dept Biol Chemistry,
Div Biochemistry, WRAIR, WRAMC, Wash. D. C. 20012
576-3528 or Interdepartmental Code 198, Ext. 3528 49
- (A) Mudd, J. Pfc, Dept Biol Chemistry,
Div Biochemistry, WRAIR, WRAMC, Wash. D. C. 20012
576-3528 or Interdepartmental Code 198, Ext. 3528 49
- (A) Christian, G. D. , PhD, Dept Clin Chemistry,
Div Biochemistry, WRAIR, WRAMC, Wash. D. C. 20012
576-3528 or Interdepartmental Code 198, Ext. 3528 49
- (A) Herman, Y. F. , MS, Dept Vet Microbiology,
Div Vet Med, WRAIR, WRAMC, Wash. D. C. 20012
576-3670 or Interdepartmental Code 198, Ext. 3670 49
- (A) Herman, R. H. , Maj, MC, Dept Biochemistry,
School Med, University of Pennsylvania,
Philadelphia, Pa. 36
- (A) Huser, H. J. , M. D. , Dept Hematology,
Div Medicine, WRAIR, WRAMC, Wash. D. C. 20012
576-3060 or Interdepartmental Code 198, Ext. 3060 49
- (A) Shields, C. E. , Maj, MC, 1964 Mil Med & Allied Sci
Course, WRAIR, WRAMC, Wash. D. C. , 20012
576-3561 or Interdepartmental Code 198, Ext. 3561 49
- (A) Ward, R. A. , PhD, Dept Entomology, Div Comm
Disease & Immunology, WRAIR, WRAMC, Wash. D. C.
20012. 576-2087 or Interdepartmental Code 198,
Ext. 2087. 49
- (A) Sheehy, T. E. , Maj, MC, Dept Gastroenterology,
Div Medicine, WRAIR, WRAMC, Wash. D. C. 20012
576-2300 or Interdepartmental Code 198, Ext. 2300 49

REPORTS. Annual Progress Report, Walter Reed Army
Institute of Research, 1 July 1963 - 30 June 1964.

PUBLICATIONS:

Campbell, W. J. and Mertz, W.: The Synergism of Insulin
and Chromium III on Mitochondrial Swelling, Fed. Proc.
Abstr. 22, 474(1963).

PUBLICATIONS:

Campbell, W. J. , and Mertz, W. : Interaction of Insulin and Chromium(III) on Mitochondrial Swelling. *Am. J. Physiol.* , 204, 1028, 1963.

Mundy, R. L. , Heiffer, M. H. , and Mehlman, B. : Mechanisms of Beta-mercaptoethylamine Induced Hypotension in the Dog. *Am. J. Physiol.* , 204, 997, 1963.

Jacobson, E. D. , Mehlman, B. , and Kalas, J. P. : Vasoactive Mediators as the Trigger Mechanism of Endotoxin Shock. , *J. Clin. Invest.* 43, 1964.

Corwin, L. M. , and Schwarz, K. : Relation of Tocopherol to Enzyme Sulfhydryl Sites. *Arch. Biochem. Biophys.* , 100, 385, 1963.

Seward, C. R. , and Corwin, L. M. : *In Vitro* Studies of the Fate of α -Tocopherol in Rat Liver Homogenates. *Arch. Biochem. Biophys.* , 101, 71, 1963.

Corwin, L. M. : Further Studies on the Regulation of Succinate Oxidation by Tocopherol. Abstracts, 6th Intern. Congr. of Nutrition, 192, 1963.

Doctor, B. P. , Connelly, C. M. , Rushizsky, G. W. , and Sober, H. A. , : Studies on the Chemical Structures of Yeast Amino Acid Acceptor RNA. *J. Biol. Chem.* , 238, 3985, 1963.

Doctor, B. P. , Mudd, J. A. : Species Specificity of Amino Acid Acceptor RNA and Amino Acyl-S-RNA Synthetases. *J. Biol. Chem.* , 238, 3677, 1963.

McCormick, G. J. and Doctor, B. P. : Countercurrent Distribution of Yeast RNA Core. *Biochem. Biophys. Acta.* 76, 628, 1963.

Christian, G. D. , Knoblock, E. C. , Purdy, W. C. : Coulometric Titration of Ammonia with Hypobromite Using Direct Amperometric End-Point Detection. *Anal. Chem.* 35, 2217, 1963.

Christian, G. D. , Knoblock, E. C. , Purdy, W. C. : A Direct Argentometric Titration of Orthophosphate. Application to Coulometric Titration , *Anal. Chem.* , 35, 1869, 1963.

Christian, G. D. , Knoblock, E. C. , Purdy, W. C. : Polarography of Selenium(IV). *Anal. Chem.* , 35, 1128, 1963.

ACCESSION NUMBER

36168

ARMY RESEARCH TASK REPORT

Continuation Sheet

PUBLICATIONS:

Christian, G. D. , Knoblock, E. C. , Purdy, W. C. :
Coulometric Determination of Protein Nitrogen, 48th Ann.
Mtg. of Fed. of Am. Soc. of Exp. Biol. , Apr 12-17, 1964,
Chicago, Ill.

DA FORM 1309R
1 June 63

PREVIOUS EDITIONS ARE OBSOLETE

Page of _____

ANNUAL PROGRESS REPORT

Project No. 3A012501B813 Title: Army Medical Basic Research
In Life Sciences

Task No. 03 Title: Biochemistry

Subtask No. 21 Title: Biochemical activity in health
and disease

Description: The purpose of this task is to provide basic information from biochemical studies with the goal of developing capabilities to undertake studies of special interest to the health of the U. S. Army. Studies involved include nutrition, development of advanced analytical techniques for separation and identification of important biochemical substances, and basic enzymological studies of infectious disease.

Progress:

Insulin-Mitochondrial Interaction - The effect of insulin and chromium on mitochondrial stability has been demonstrated in this lab as well as in other laboratories. At present using the mitochondrial membrane as a model the effect of different insulin and chromium concentrations on various substrates is being investigated. It has been demonstrated that mitochondria behave differently in the presence of different substrates. An attempt is underway to show the difference in oxygen uptake by mitochondria under the same conditions of insulin and chromium. Preliminary results indicate that there is a considerable difference in succinate oxidation, which is not seen in α -ketoglutarate, malate or other substrates when insulin & chromium are added to the medium. This study is still underway.

Study of Pressor Substances- Analyses of pressor substances is being done to provide diagnostic assistance to service hospitals whose facilities are not adequate for the performance of these complicated techniques. In addition, the role of pressor substances in various types of trauma is still not well known and collaborative studies with other investigators are being carried out. New analyses are developed and adapted as time permits. Some 50 samples of possible cases of pheochromocytoma have been analyzed; about equal to last year. In addition, collaboration was given the Cardiovascular Clinical Research Center of Temple University in an attempt to determine a possible site of a tumor believed to be along the sympathetic pathway by analyzing samples obtained by catheterization of the vena cava. Since there have been hints in the literature that the pressor amines may be involved in cholera, preliminary studies have been started with the Division of Communicable Disease and Immunology, to measure plasma catechols, histamine and serotonin in monkeys infected with shigella. The method of Oates, et al, Clin Chim Acta 7, 488(1962) for urinary histamine was evaluated and put into use. Similarly, a method for 5-Hydroxyindole acetic acid (5HIAA) the major urinary metabolite of serotonin was established. (Udenfriend, S. et al, Methods of Biochemical Analysis, Vol 6, 113, 1958.)

Lipid-Soluble Vitamins - It has been shown that in the presence of added NAD, the oxidation of succinate by liver mitochondria from rats deficient in vitamin E is not maintained. The decline of oxidation can be prevented by *in vivo* or *in vitro* addition of the vitamin. Supporting previous indirect evidence, it has now been demonstrated that the decline of oxidation is due to an accumulation of oxaloacetate (OAA) which is twice that of the supplemented mitochondria. Tocopherol has no effect on the removal of OAA or the accumulation of pyruvate and citrate. In the absence of tocopherol 3 times as much CO_2 is formed from succinate oxidation. It was therefore concluded that tocopherol inhibits the formation of OAA, a potent inhibitor of succinate oxidation, rather than accelerate its removal. It was shown that tocopherol stimulated acetoacetate reduction by succinate. Acetoacetate reduction has been used as a measure of the reduction of NAD. From such evidence, it was tentatively concluded that since tocopherol stimulates the reduction of NAD for succinate, less NAD is available for the oxidation of malate to oxaloacetate. Thus with decreased OAA formation less inhibition of succinate oxidation occurs. Subsequent experiments have indicated that the previous results may be a result of an effect of tocopherol on the transport of NAD in the medium into the mitochondria. It is now felt that tocopherol stabilizes the mitochondrial membrane and prevents the entry of NAD to the inside of the mitochondria. This is borne out by results obtained by adding calcium ions to the medium, an unknown to breakdown permeability barriers in mitochondria. Calcium has no effect on succinate oxidation in the absence of NAD, but severely inhibits oxidation when NAD is added even in the presence of tocopherol. This tends to implicate the action of tocopherol in the prevention of NAD entry into the mitochondria, thereby decreasing the formation of OAA and inhibition of succinate oxidation.

Biochemical Activity of Endotoxin - In a joint project with Applied Immunology, the nature of the endotoxin-inactivating principle of guinea pig liver was ascertained. Endotoxin is a lipopolysaccharide obtained from cell walls of many gram negative bacteria. A current problem in the field has been whether the lipid or the polysaccharide is the toxic agent of endotoxin. We have found that guinea pig liver homogenates inactivate endotoxin and have two pH optima, one of which can be isolated in mitochondria. Acetone powders of the mitochondria can be stimulated by ATP and NAD, and the mitochondria can also be activated by malate. With this information and a knowledge of the chemical nature of the substrate, it was concluded that the enzymes involved in the inactivation of endotoxin were those that activate and oxidize fatty acids. Thus, it was further concluded that the lipid moiety of endotoxin is necessary for toxicity.

Biochemistry of trans Isomers of Unsaturated Fatty Acids - It was previously reported that experiments performed in this study showed that fat from rat erythrocyte membrane contained about 50 times as much trans fatty acid than did depot fat. A literature search revealed that the phosphorus-oxygen-carbon bond system

absorbed strongly in the infrared region of 10.36 microns where the trans double bond is measured quantitatively. This would explain the strong peaks presumed to be due to trans in the erythrocyte extracts, since the phospholipid content of depot fat is much lower than that of any other body tissue. A method was developed to eliminate this interfering absorption peak; fatty acids were transesterified by treating crude lipid extracts with boron trifluoride-methanol reagent. The resulting solution was partitioned between petroleum ether and water in a separatory funnel. The methyl esters of the fatty acids were soluble in the petroleum ether layer; the interfering glycerate-choline phosphate portion was soluble in the aqueous layer. The petroleum ether layer was isolated, evaporated, and used in infrared determinations. It was determined that the methylation technique has no deleterious effect on the absorption due to trans. The effect of cholesterol (present in rather high concentration in biological membranes) or the trans absorption peak was studied. Interference was found at cholesterol concentration of 0.05 M or greater. A digitonin precipitation technique was adapted to remove high concentrations of free cholesterol from lipid extracts.

Rats fed for 3 months on diets containing up to 20% trans fatty acids showed no obvious deleterious effects; their average weight gain and susceptibility to liver necrosis (in the absence of dietary Vitamin E and Factor 3) were not significantly different from controls fed trans-free or low trans diets.

Experiments are now in progress to determine the effect of Factor 3 (biologically effective selenium) on the deposition of trans fatty acids in erythrocyte membrane, liver mitochondria, and depot fat. Work is being planned at the present time to study the effect of high tissue concentrations of trans fatty acids on membrane permeability in the erythrocyte and the mitochondrion.

Methods of Protein Separation - New methods and improved techniques are being investigated in connection with protein, lipoprotein and mucoproteins levels in serum. The purpose of this work is to provide more efficient support to medical diagnosis and metabolic studies.

Disc Electrophoresis - This technique resolves serum proteins into more components than any other electrophoretic method. Its extreme sensitivity requires meticulously clean glassware and careful watch over any gel deterioration. Our work was held up several months by the appearance of bands in the gels that were supposed to be blank. The trouble was eventually traced to the glycine buffer.

Mucoproteins - A relatively quick method for characterizing the serum mucoprotein pattern has been devised. 0.2 ml serum is treated with 0.06 ml 2N HClO₄ and then centrifuged. The supernatant is treated with 0.03 ml 2N KOH and recentrifuged. 0.1 ml undiluted upper gel is added to the supernatant and the combined liquid applied

to the column above the spacer gel. With normal sera, only 2 well defined protein bands result. When the sera from 2 cancer patients who had received whole body irradiation were treated in like manner 5 or 6 bands resulted. It is planned to continue this investigation and also to extend it to a study of various diseases.

Lipoproteins as a biological dosimeter of irradiation in man-
The sera from several people who had received whole body irradiation were analyzed for lipoproteins. Contrary to some previous findings no marked lipoproteins response was noted.

Lipoprotein response to diet- The sera from people on a strict vegetable diet were compared to people with no diet restriction. Total cholesterol as well as low density lipoprotein cholesterol was determined. Twenty-two vegetarians had an average total cholesterol of 203 mg% and low density lipoprotein cholesterol of 122 mg%. Seventeen non-vegetarians had an average total cholesterol of 227 mg% and a low density lipoprotein cholesterol of 156 mg%. The two groups were significantly different with respect to low density lipoprotein cholesterol at the 5% level. Total cholesterol levels were not significantly different.

Synthesis of Trypsin Inhibitors- A continuing study to synthesize organophosphorous compounds whose structural changes will produce non-toxic inhibition toward certain enzyme systems.

A series of O-p-nitrophenyl O-ethyl W-chloroalkylphosphonates with the alkyl group varying in length from 4 to 7 carbons were synthesized. Their inhibitory activity against red cell human acetylcholinesterase, trypsin and chymotrypsin was compared with that of a series of O-p-nitrophenyl, O-ethyl alkyl and phenylalkylphosphonates whose inhibition against the same enzymes had been tested previously.

Of the four chloroalkyl compounds tested, the 6-chlorohexylphosphonate showed peak anti-chymotrypsin activity, but gave minimum anti-trypsin activity. The greatest activity against acetylcholinesterase came with the 7 chloroheptylphosphonate. This pattern of inhibitory activity differed distinctly from that reported for the corresponding alkylphosphonates.

When the number of carbons in the alkyl chain is between 4 and 7, neither the number of the atoms in the alkyl chain, nor the presence of a chlorine atom on the terminal carbon affects appreciably the rates of the reaction of these phosphonates with water. However, it has been demonstrated, that both factors affect the reactivity of these compounds with enzymes. The decrease in activity against cholinesterase for the lower alkyl chain member is attributed to the repulsion

of the strategically placed chlorine atom by the anionic site of the enzyme. As the carbon chain is lengthened, the effect of the chlorine atom is progressively lessened and the activities of alkyl and chloroalkyl compounds tend to approximate each other.

It has been postulated that a negative charge is present in chymotrypsin close to one of the loci for attachment of the aromatic group. The activity of the chloroalkylphosphonates suggests that the negative charge is too far from binding site of the alkyl chain to interact with the terminal chlorine atom. This effect seems rather to be due to its size since it seems to act similarly to an added methylene group.

The minimum in antitrypsin activity found with the 6-chlorohexylphosphonate contrasts with the optimum given by the corresponding hexylphosphonate. Positively charged amino acids, are required in the preferred substrates for trypsin suggesting that this molecule possesses an anionic binding site at a definite distance from the esteratic site. If this is so, the minimum antitrypsin activity given by the 6-chlorohexyl compound might be due to the repulsion of the chloro group by this anionic site.

The Chemical Structure of Nucleic Acids- One of the most vital functions of the cell is to replicate. The cell is not capable of performing this if its protein synthesizing mechanism is impaired. It is well known that nucleic acids control and regulate the protein synthetic function of the cell. Thus it is vital to study the structure of Nucleic Acids and its co-relation to biological function. These studies in turn will furnish better knowledge regarding the irregularities of cellular metabolism observed during the diseases and infections by bacteria & viruses.

The role of transfer-RNA in protein synthesis is to transfer the activated specific amino acid to the site of protein synthesis. The genetic information regarding the sequence of amino acids in protein molecule resides in the sequence of nucleotides in S-RNA. Using counter current distribution procedures alanine- and tyrosine -S-RNAs were purified. Further improvements in these procedures have made it possible to obtain these RNAs in relatively large quantities, thus making it possible to study their structures in detail. The pancreatic RNase digest of these two RNAs showed the great variations in their nucleotide sequences. The ribonuclease, digests are under investigation. The information obtained from these two studies will make it possible to assemble some of the detailed sequences of their nucleotides. It has been shown that genetic code is degenerate for many amino acids. In order to study this phase of protein synthesis it was decided

to study the nature of degeneracy in serine S-RNA from the yeast. S-RNA from yeast contain two serine-RNAs. Using countercurrent distribution techniques two serine -RNAs were separated and purified.

Further studies of these two RNAs revealed that the rate and extent of serine incorporation with rat liver or yeast amino activating enzymes is the same for both RNAs. *E. coli* enzymes is inactive towards both RNAs. The transfer of serine using poly Uc C2:1 or 1:4 ratio showed no difference towards the activity of these two RNAs. The chemical studies are under progress at the present.

Using paper or column chromatography and/or electrophoresis it is possible to separate nucleotides up to the chain lengths of four. The existing methods are incapable of resolving the high oligonucleotides.

A solvent system has been developed in which one can fractionate oligonucleotides of the same chain lengths using countercurrent distribution. Using these techniques it has been possible to identify the existence of two hexa-nucleotides in mixtures of S-RNA from yeast. These two hexa-nucleotides have (1) 2 Ap, 3 Ap & cp (2) 3Ap, 2 crp and cp. Similarly hepta-nucleotides consisting of (1) 2Ap; 4 crp and Cp (2) 4Ap; 2 crp & cp and a mona-nucleotide containing 5 Ap. 3 crp and cp have been identified.

Applications of Electroanalytical Chemistry - The studies described are an extension of the applications of electroanalytical chemistry to the determination of biologically and clinically important substances.

a. Polarography of selenium in highly acid medium. Selenium (IV) in acid medium gives two polarographic waves at the dropping mercury electrode (D. M. E.). The first is a four-electron reduction resulting in formation of mercuric selenide by reaction with the electrode. The second wave corresponds to reduction of the mercuric selenide. In highly acid medium, this wave slowly disappears on standing in the presence of mercury. This has been determined to be due to slow acid dissolution of mercury (e.g. from the electrode) which precipitates the intermediate selenide ions formed at the electrode surface. Formation of mercuric selenide on the electrode is prevented and therefore the second wave is eliminated.

b. Polarographic Determination of Selenium in Biological Materials. A direct polarographic determination of selenium in acid digests was developed. For increased sensitivity, however, the selenium is isolated as the element or by extracting the diaminobenzidine complex into an

organic solvent. The complex can be back-extracted into 2M perchloric acid, where it is polarographically reducible; two selenium waves are found. These correspond to four and two electron reductions, respectively. Only the first is completely reversible. As little as 0.2 microgram of selenium can be determined. All methods developed require two hours or less for an analysis.

c. The Amperometric Titration of Total and Inter-chain Disulfide Bonds in Gamma Globulin. The total disulfide contents of gamma globulins can be determined by amperometric titration of with mercuric chloride in the presence of sulfite and 8M guanidine hydrochloride. Results have been compared with those obtained by other methods. The determination of inter-chain disulfide bonds by the method of Cecil and Wake was investigated. A constant number of disulfide bonds was reduced over a large range of sulfite to protein ratio. Beyond a certain limiting ratio, more bonds were reduced; the nature of these bonds is uncertain.

d. The Role of Disulfide Bonds in the Complement-Fixing and Precipitating Properties of 7S Rabbit and Sheep Antibodies. The number of total disulfide bonds in rabbit and sheep 7S gamma globulin, before and after treatment with 2-mercaptoethanol has been measured by amperometric titration. The reduction of disulfide bonds in relation to the decrease of the complement-fixing and precipitating ability of 7S rabbit and sheep antibody was investigated. The complement fixing efficiency of 7S rabbit gamma globulin could be diminished by no more than 90%. This was associated with the reduction of only seven disulfide bonds, including one inter-chain disulfide bond. The reduction of seven disulfide bonds in 7S sheep gamma globulin was associated with 64% decrease in complement-fixing efficiency, while reduction of ten to eleven disulfide bonds decreased the complement-fixing efficiency by 90%. Reduction of more disulfide bonds were not associated with any further decrease in complement-fixing efficiency. The reduction of more than ten to eleven disulfide bonds in 7S rabbit and sheep gamma globulins was associated with a decrease in precipitating ability. Therefore, the disulfide bonds which are more labile to mercaptan reduction appear to be associated with complement fixation, while the disulfide bonds which are more resistant to mercaptan reduction appear to be associated with precipitating ability. One easily reduced inter-chain disulfide bond ($S-S_{(1)}$) appears to be important for the complement fixing efficiency of 7S rabbit antibody. The integrity of the same bond is essential for the precipitating ability of 5S rabbit antibody and may also be important for its complement fixation.

e. Coulometric Determination of Urea Nitrogen. A coulometric titration of ammonia with electrogenerated hypobromite using a direct amperometric end-point detection was previously reported. This ammonia titration has been used to determine urea nitrogen following urease hydrolysis. The ammonia can be directly in urine samples. With blood samples it must be separated by microdiffusion before titrating, because of high titration values of traces of protein.

f. Coulometric Determination of Protein Nitrogen. This method is based on titration of ammonia resulting from micro-Kjeldahl digestion of protein samples. The ammonia can be titrated without separation if a mercury catalyst is used. Serum samples as small as one microliter can be taken for analysis. Results agree favorably with those obtained by standard Kjeldahl procedures.

g. Coulometric Determination of Blood Ammonia. The ammonia (ca. 0.5 microgram or less) in a one ml sample is separated by microdiffusion. It is titrated coulometrically using a new sensitive amperometric end-point detection. Excess of the titrant (hypobromite) is generated and the linear increase in amperometric current is recorded. The sample is then added and the decrease in the amperometric current is measured. This is converted to the corresponding time equivalent by using the recorded linear amperometric current increase above. At present, the method is empirical (must be compared with standard). However, further work is being conducted in hopes of making the titration absolute.

h. Coulometric Titration of Proteins. A direct titration of native proteins was developed. The titrant is coulometrically generated hypobromite and the end-point is detected amperometrically. The variables of the titration must be kept constant for both samples and standards. These include solution volume, sample size, generating rate, and stirring rate. From 2 or to 2 microliter serum samples have been titrated. Serum albumin and globulins give approximately the same titration value per mole. Therefore, the total number of mols of protein in serum may be determined without separation of the proteins. Probable reactions of hypobromite with proteins include oxidation of disulfide bonds, amino groups, and phenylhydroxy groups.

i. Automatic Recording of Dual-Electrode Amperometric Currents in Coulometric Titration. Most precision coulometric procedures employing dual-electrode amperometry list manual recording of the amperometric current. Many of these should be adaptable to automatic recording. By this method, any type of titration curve could be recorded and precision measurements could be made as with manual procedures.

Several of the common coulometric titrations employing dual-electrode amperometry have been investigated to illustrate the reliability of automatic current recording. Included are titrations of arsenic (III) with electrogenerated iodine, bromine, and chlorine, titration of iodide ion with bromine and cerium (IV), thiosulfate with iodine, and iron (III) with chlorocuprous ion. Results approach or exceed the manual measurements in accuracy and precision and the titration time is decreased to a fraction of the time required for manual titrations.

j. Coulometric Determination of Hydrogen Peroxide. An iodometric procedure was developed. Hydrogen peroxide oxidizes iodide ion to iodine in the presence of a molybdenum catalyst. Excess standard thiosulfate solution is added and the excess is back-titrated with electrogenerated iodine. A wide range of pH (ca. 0 to 7.5) has been investigated. Compensation of errors in acid medium was determined. The method has been applied to the determination of 2.6 mg to 0.1 μ g of hydrogen peroxide. This titration is presently being investigated for the determination of biologically important substances which can be converted to hydrogen peroxide (e. g. glucose and uric acid).

The carbohydrate metabolism of non-infected and malaria-parasitized non-nucleated and nucleated red blood cells of selected species -
As a first step the carbohydrate metabolism, particularly the pentose phosphate pathway, of sheep red blood cells which are reported to be deficient in the enzyme glucose-6-phosphate dehydrogenase (G-6-PD), but resistant to the effects of the antimalarial compound, primaquine, was investigated. As normal control, dog red blood cells were used. However, dog red blood cells are noted for their poor storage qualities. The nature of the supposed sheep red blood cell G-6-PD deficiency and primaquine resistance was studied by measuring the production of $^{14}\text{CO}_2$ from 1- ^{14}C -glucose of intact RBC's and by the spectrophotometric assay of enzymes on the hemolysates of the same RBC suspension.

It has been found that intact sheep red blood cells produce carbon dioxide from 1- C^{14} -glucose especially in the presence of various substances. Assay of the enzymes in hemolysates show that there is a marked deficiency of glucose-6-phosphate dehydrogenase and 6-phosphogluconic acid dehydrogenase as compared to man. Table 1 indicates the amount of C^{14}O_2 produced from 1- C^{14} -glucose.

Table 1. The production of C^{14}O_2 from 1- C^{14} -glucose. The C^{14}O_2 is expressed as the percent of the initial radioactivity of the 1- C^{14} -glucose used. Values are averages of duplicate determinations.

Animal No.	Vol of Packed RBCs (ml.)	Total Vol.	Additions		$C^{14}O_2$ Produced
			Substance	Conc. (mM)	
319	0.2	3.0	None	---	11.6
319	0.5	3.0	None	---	17.4
335	0.2	3.0	None	---	12.6
335	0.4	3.0	None	---	23.8
319	0.2	3.0	TPN	2	34.4
319	0.5	3.0	TPN	2	40.4
335	0.2	3.0	AD-DPN	2	30.0
335	0.4	3.0	AD-DPN	2	50.6

Other studies have shown inhibition of the $C^{14}O_2$ production by a sulfhydryl inhibitor, namely, N-ethylmaleimide and stimulation of the system by reduced glutathione.

Enzyme assays are shown in Table 2 and comparable values in normal human subjects are also given.

Table 2. Values of various enzymes of the pentose phosphate pathway in sheep and man. Values are given as units of enzyme activity/gram of hemoglobin. One unit of enzyme activity equals a change of .001 optical density units/minute at 340 mu in a 1 cm. light path.

Sheep No.	Glucose-6-Phosphate	6-Phosphogluconic Acid
	Dehydrogenase	Dehydrogenase
319	1430	---
332	1739	---
346	2110	< 530
62	1250	0
<u>Human Subjects.</u>		
1 (YH)	8400	---
	12190	---
	12310	6670
2 (CC)	10400	5200
3 (C)	10435	4780
4 (RH)	11250	5000

In order to determine why intact sheep red blood cells were active in the metabolism of 1-C¹⁴-glucose but enzyme activity was so low in hemolysates, the activity of sheep hemolysates in metabolizing 1-C¹⁴-glucose was studied. Table 3 shows studies done on intact sheep red blood cells and sheep hemolysates.

Table 3. C¹⁴O₂ production by intact sheep red blood cells and sheep hemolysate. Details are as for Table 1.

Vol. of Packed RBCs (ml.)	Total Vol. (ml.)	Additions		C ¹⁴ O ₂ Production	
		Substance	Conc. (mM)	Intact RBCs	Total Hemolysate
0.2	3.5	None	---	23.6	1.4
0.2	3.5	ATP	2	26.0	1.6
0.2	3.5	TPN	2	33.0	28.3
0.2	3.5	DPN	2	32.0	40.2
0.2	3.5	GSH	2	49.0	1.5

As can be seen in intact red blood cells, some stimulation of C¹⁴O₂ production occurred with TPN and DPN and particularly with GSH. In hemolysates very little C¹⁴O₂ was produced, unless TPN or DPN were present and GSH was very poor in effecting stimulation. Obviously some change occurs on hemolysis which prevents the proper functioning of the pentose pathway enzymes. Since enzyme assays are done on hemolysates, this accounts for the finding of apparent enzyme deficiency.

Considerable difference in the activity of dog red blood cells was found as compared to man and sheep red blood cells. Table 4 indicates the results obtained.

Table 4. C¹⁴O₂ produced by intact dog red blood cells. Details are as for Table 1.

Dog No.	Vol. of Packed RBCs (ml.)	Total Vol. (ml.)	Additions		C ¹⁴ O ₂ Production
			Substance	Conc. (mM)	
6z9	0.2	3.0	None	---	0.1
6z9	0.5	3.0	None	---	0.4
6z9	0.2	3.0	TPN	0.5	0.4
6z9	0.2	3.0	TPN	1.0	0.3
6z9	0.2	3.0	TPN	2.0	0.3

The results of Table 4 are typical of many such experiments. GSH was poorly stimulatory. Assay of the enzymes in hemolysates gave values which are shown in Table 5.

Table 5. Assay of enzymes of the pentose phosphate pathway in dog hemolysates. Details are as in Table 2.

Dog No.	Glucose 6-Phosphate Dehydrogenase	6-Phosphogluconic Acid Dehydrogenase
A207	6500	---
A290	8600	---
A265	8700	---
Z84	8230	---
A242	16840	5260
A940	11000	1900

Thus, intact dog red blood cells metabolize 1-C¹⁴-glucose poorly via the pentose phosphate pathway though the enzymes are quite active in hemolysates. Studies of 1-C¹⁴-glucose metabolism by hemolysates, however, have shown poor utilization of 1-C¹⁴-glucose. However, when ATP and TPN were added together, this poor utilization of 1-¹⁴C-glucose by hemolysates was corrected.

Preparatory to the study of the carbohydrate metabolism of non-infected and malaria parasitized nucleated RBC's, base line data was obtained on the ¹⁴CO₂ production from 1-¹⁴C-glucose by RBC's of chicken, goose and duck. The same methods employed in the 1-¹⁴C-glucose metabolism of sheep and dog red blood cells were followed with a slight modification in the centrifugation rate for nucleated cells being reduced to 1800-2000 rpm's for ten minutes. In Table 6 the results are summarized.

Table 6. The ¹⁴CO₂ production of intact red blood cells and total hemolysates from chickens, geese and duck. The data is expressed as in Table 1.

Species	No. of Animals	Intact Red Blood Cells		Total Hemolysate	
		No Additions	TPN	No Additions	TPN
Chicken	pool of 3	0.2	0.4	15.2	40.5
	1	1.0	1.3	21.5	14.0
	pool of 5	0.2	0.5	19.2	32.0
Goose	1	0.1	0.2	0.9	39.5
	1	0.4	0.7	3.9	51.3
	1	0.1	0.6	1.3	39.5
	1	0.1	0.3	3.2	37.2
Duck	1	0.3	0.8	0.6	23.1

In the performance of the comparative studies between non-infected and malaria parasitized chicken red blood cells, microhematocrits were done on all cell types and percent parasitemia of red blood cell populations were also made by counting 1500 RBC on Giemsa-stained blood films. Chickens were infected with Plasmodium gallinaceum. Blood was obtained by cardiac-puncture and heparinized. RBC's were washed 3 times in Kreb's Ringer, pH 7.2, and 0.2 ml of packed cells were used in the experimental flasks.

Table 7.

Species RBC Intact	Hem- atocrit	Per Cent of Parasitemia	Additions*		
			None	TPN	ATP
*Pool of 3 Chicken, Normal	n.d.		0.22%	0.43%	
*Pool of 3 Chicken, Infected	n.d.	80.0%	21.21%	21.64%	
**Pool of 2 Chicken, Infected	n.d.	84.5%	14.44%	19.23%	
***Chicken, Normal	25%		0.96%	1.25%	1.44%
***Chicken, Infected	24%	14.0%	4.60%	5.21%	3.25%
***Chicken, Infected	18%	90.0%	13.83%	19.39%	11.49%
***Chicken Infected	17%	90.0%	17.88%	19.39%	9.31%

* 24,915.6 dpm of 1-¹⁴C-Glucose per flask, initial.

** 24,907.2 " " " " " " " " "

*** 21,550.4 " " " " " " " " "

Red cells were infected with Plasmodium gallinaceum.

Since malaria also affects primate red blood cells and since there is an apparent species specificity in that a particular plasmodium specie affects a specific primate red blood cell, although in a few cases plasmodia will affect more than one primate species, the baseline information on the peculiarities of the glucose metabolism of the particular non-human primate RBC species were obtained concurrently with information from studies on the 1-¹⁴C-glucose metabolism of the RBC's of Rhesus monkeys infected with Plasmodium cynomolgi bastiani by vector transmission. At different stages of parasitemia, the ¹⁴CO₂ production from 1-¹⁴C-glucose by the red blood cells of two infected rhesus monkeys was compared with that of erythrocytes of

non-infected animals. Preliminary results show a marked difference between the two groups, the degree of difference being roughly correlated with the parasitemia rate. To date the wide variety of non-human primates studied include orang-utan (Pongo pygmeus), chimpanzee (Pan satyrus), gorilla (Gorilla gorilla), baboon (Papio cynocephalus), rhesus monkey (Macaca sp.), Black ape (Cyno-pethicus niger) and pigtail monkeys. On the basis of these preliminary results, a more definitive study is planned.

Course of experimentally-induced malaria in rats with a folic acid deficiency - The microbiological assay for the determination of serum and plasma folates was standardized with materials kindly contributed by Dr. Victor Herbert whose reported method is rigidly followed. The test organism is Lactobacillus casei. Minute amounts in the range of 10^{-11} grams/ml of serum or plasma folates can be assayed microbiologically, so that folate levels can be determined at any point to ascertain conditions of either controlled or natural folic acid deficiencies. Ten young rats were individually caged and placed on a folic-acid deficient diet. Ten rats of the same age and weight on a normal diet were used as controls. Plasma was obtained by puncture of the orbital sinus with a heparinized micro-capillary tube. This was done weekly and the plasma folate levels determined. Mouse red blood cells infected with Plasmodium berghei was used as inoculum to prepare the first passage material of infected rat RBC's. It was possible at first to infect very young rats with a large dose of P. berghei. However, considerable time was required to make the rats folic-acid deficient, so that by the time they were borderline deficient, they had increased in weight and size and became refractory to the injection of plasmodia infected rat RBC's. Plasmodium berghei exists only in the erythrocytic stage, hardly ever is found in the liver tissue phase, or is it mosquito-transmissible. Thus, it appears that Plasmodium berghei is not the organism of choice. More definitive studies are being planned employing the avian malaria, Plasmodium gallinaceum.

Field Studies -

a. In May and June, 1963, personnel of the Division of Biochemistry were sent to Bangkok to set up a method for the analysis of Chloroquine in blood. Equipment was purchased including an Aminco-Bowman Spectrophoto fluorometer and a modified Brady fluorometric procedure was set up. In the ensuing four months several hundred serum chloroquine levels were determined. In many cases serum levels as high as 400 to 500 γ gm/liter were found and high levels maintained for 36 to 72 hours. Many of these sera were from individuals who had Plasmodium falciparum and who subsequently relapsed. A definite resistance of P. falciparum

to high serum levels of chloroquine was established. Further studies are being conducted to determine distribution of chloroquine in the animal body.

b. A team was sent to Bangkok to investigate various biochemical parameters of the Thai population with respect to multivitamins, and multivitamins + Vitamin E. A double blind trial using Placebo, and the two vitamin preparations was conducted on 300 Thai volunteer subjects ranging in age from 2 months to 72 years. Each subject was given a physical examination including EKG, and blood and urine specimens were collected. Serum enzyme levels, Vitamin E levels, total protein, and protein electrophoresis were determined. A complete hematology work-up and urine creatine-creatinine ratios were performed. Both the physical examinations and laboratory tests were performed on each subject before and after the two-week drug trial.

Even though the serum Vitamin E level of the Thai population is lower (approx. 6.8 γ gm/ml) than that found in the United States, no evidence was found which would imply a deficiency exists. Those individuals receiving Vitamin E had elevated Vitamin E levels on the second examination, but none of the other measurements reflected this rise. Full evaluation of the program is incomplete pending assembly and evaluation of all data collected. Obvious Vitamin B₂ deficiency was observed, and the angular lesions healed rapidly upon Vitamin administration. The pattern of EKG appears to differ from that seen in western subjects, and is possibly due to the smaller body size of the adult Thai. Serum enzymes, total protein and hematology values were not remarkable except for a large amount of eosinophilia which is to be expected in an area of high incidence of parasitic infestations.

Summary and Conclusions:

The effect of chromium (III) in the presence of insulin on mitochondria has been investigated. The combined action on various measures of mitochondrial stability and activity are still under investigation. Certain differences in substrate oxidation have been noted and are being investigated further.

A research team of 8 persons went to Bangkok to study various aspects of vitamin metabolism. The data has been accumulated and is being analyzed. In a separate effort, an investigation of the suppression of malaria was also carried out at the request of SEATO Medical Laboratory with personnel being provided on temporary duty to Bangkok.

Some 50 specimens have been received requesting epinephrine and norepinephrine assays on blood plasma as an aid in the diagnosis of pheochromocytoma.

Active collaborative studies have been done with the Department of Neuroendocrinology and the Division of Communicable Diseases and Immunology involving plasma catechols, serotonin and histamine, and with the Medical Unit, Fort Detrick involving plasma and urinary catecholamines. A urine histamine method was evaluated and put into use. Similarly a method for 5-hydroxyindole acetic acid (5H-IAA) the major metabolite of serotonin was established.

It has been shown that in the presence of NAD, the oxidation of succinate by liver mitochondria from rats deficient in vitamin C is not maintained. The decline of oxidation can be prevented by in vivo or in vitro addition of the vitamin. The inhibition has been shown to be due to an accumulation of oxaloacetate (OAA). Tocopherol prevents the accumulation of OAA by allowing greater reduction of NADH to occur, thereby shifting the equilibrium between malate and OAA towards malate.

In a joint project the nature of the endotoxin inactivity principle of guinea pig liver was ascertained. The enzymes were shown to be most probably those involved with fatty acid activation and oxidation. Such a result indicates that the lipid moiety of the lipopolysaccharide is required for the toxicity of endotoxin.

A transesterification procedure has been developed to eliminate an interfering infrared absorption peak in the determination of trans isomers of unsaturated fatty acids in crude lipid extracts. It has also been found necessary in some cases to reduce the concentration of free cholesterol in these extracts by means of digitonin precipitation. Rats fed high concentrations of trans fatty acids seem to be grossly unaffected by them.

The disc electrophoretic technique has been perfected and is now ready for application. Resolution of low density lipoproteins has not been successful to date but further work is being done. The determination of serum mucoproteins by means of disc electrophoresis is quite promising. Cancer patients that have received whole body irradiation displayed mucoprotein patterns radically different from normals. People of a vegetable diet had lower levels of low density lipoproteins than non-vegetarians.

A continuation of the synthesis of organophosphorous compounds is being made based on the previous findings that structural changes in the organophorous compounds affect the inhibitory activity of certain enzyme systems, namely, trypsin, chymotrypsin, and acetylcholinesterase.

An improved countercurrent distribution procedure enables the isolation of purified alanine and tyrosine RNA in relatively large amounts. The chemical structures of these RNAs has been studied. Two Serine acceptor-RNA from yeast have been separated and purified. The physical, chemical and biological basis of differences in these two RNAs has been studied. Methods have been developed to separate and purify the oligonucleotides of same chain length.

The study of the polarographic characteristics of selenium was continued. A polarographic procedure has been developed to determine as little as 0.2 microgram of selenium in biological samples. Coulometric determinations of urea nitrogen in blood and urine, protein nitrogen, blood ammonia, and hydrogen peroxide have been developed. In addition, a coulometric titration of native proteins is described. Automatic recording of amperometric end-point in coulometric titrations was investigated. Amperometric titration of disulfide bonds in gammaglobulins was investigated and has been applied to the determination of the role of disulfide bonds in the complement fixing and precipitating properties of 7S rabbit and sheep antibodies.

As little as 0.2 microgram of selenium in biological samples may be determined polarographically. Urea nitrogen, protein nitrogen, blood ammonia, hydrogen peroxide, and nature proteins may be determined coulometrically. Total and inter-chain disulfide bonds in gammaglobulin may be titrated amperometrically. The role of disulfide bonds in the complement-fixing and precipitating properties of 7S rabbit and sheep antibodies can be determined employing this titration.

The glucose metabolism of normal and parasitized erythrocytes from various species was studied to provide clues on biochemical determinants in malaria infections. Attempts were made to study the course of malaria infections in folic acid deficient rats.

The supposed sheep red blood cell G-6-PD deficiency was not a deficiency when measured by the production of $^{14}\text{CO}_2$ from 1- ^{14}C -glucose by intact red blood cells. The spectrophotometric assay for G-6-PD of the supernatant fluid of hemolyzed red blood cells showed

low activity which was labile by virtue of denaturation of the enzyme by dilution. The difference in the methods of measuring G-6-PD accounted for the supposed G-6-PD deficiency. Sheep red blood cells are not lacking in G-6-PD, thus, their resistance to primaquine. Dog red blood cells which were used as controls and presumably thought to be normal were apparently abnormal in that they were primaquine sensitive and required both ATP and TPN together in the production of CO_2 from glucose.

Base-line data in the 1- ^{14}C -glucose metabolism of chicken, goose and duck red blood cells were obtained. There was a statistically significant difference in the production of $^{14}\text{CO}_2$ from 1- ^{14}C -glucose between nucleated red blood cell species and human erythrocytes. There was a marked increase in the metabolism of 1- ^{14}C -glucose to $^{14}\text{CO}_2$ by the malaria infected chicken erythrocytes. The results of comparative studies between parasitized and non-infected red blood cells showed that additions of TPN and ATP usually stimulated the utilization of the glucose only in total hemolysates. The supernatant fluid contained virtually all the activity, while none was found in the stroma. Plasmodium gallinaceum apparently does not have a pentose phosphate pathway. The pentose phosphate pathway of the host erythrocyte is apparently used by the malaria organism.

Base-line data on the peculiarities of the glucose metabolism of a wide variety of non-human primates were obtained. There was a marked difference in the $^{14}\text{CO}_2$ production from 1- ^{14}C -glucose between normal and malaria infected red blood cells of the Rhesus monkey.

The rodent parasite, Plasmodium berghei, is apparently not the malarial organism to employ in studies to compare the course of infection between normal and folic-acid deficient rats.

ARMY RESEARCH TASK REPORT		REPORTS CONTROL SYMBOL CSCRD-6(R2)
ACCESSION NUMBER 36169	PROJECT, TASK, OR SUBTASK NO. 3A012501B8130501	
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D.C. 20315	2. FUNDING AGENCY Army Medical R&D Command Office of The Surgeon General Washington, D.C. 20315	
3. CONTRACTING AGENCY NA	4. CONTRACTOR AND/OR GOV'T LABORATORY A Walter Reed Army Inst of Rsch Walter Reed Army Medical Center Washington, D.C. 20012 723-1000 Ext 3552	
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Becker, E.L., Ph.D., M.D., Division of Communicable Disease & Immunology Dept of Immunochemistry, WRAIR, WRAMC, Washington, D.C. 20012 576-3665 or Interdepartmental Code 198, Ext 3665-See Continuation sheet		
6. TITLE OF: PROJECT <input type="checkbox"/> TASK <input type="checkbox"/> SUBTASK <input checked="" type="checkbox"/> Antigen-antibody reactions in vivo and in vitro (U)		
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964		
8. RESUME (U) Various phosphonates were studied for inhibition of guinea pig first component of complement; C'1a and antigen-induced histamine release from guinea pig lung. The pattern of inhibition of C'1a was more similar to trypsin than chymotrypsin or anticholinesterase. The inhibition of histamine release was similar to that of C'1a. An amperometric titration was developed and applied to the study of disulfide bonds of gamma globulin. Aromatic amino acid derivatives competitively inhibit formation of intermediate (EAC'1a,4,2a,3cb) from EAC'1a,4,2a and EAC'1a,4,2a,3c. Human RLS restores hemolytic activity to an RLS but not its ability to support histamine release. Treatment of antisera with pepsin or reducing agents does not affect their histamine releasing ability. Heparin in high concentration prevents activation of permeability globulins in human plasma but not in serum. Kallikrein is more resistant to inhibition by heparin than Ff/dil. Early chicken antisera bleedings gave denser Ouchterlony bands with 1.5M NaCl than with 0.15M NaCl; the reverse was true of later bleedings. Thermodynamic studies reveal a heterogeneity of binding affinities of the anti-B antibody present in sera of unstimulated individuals. Family studies of red cells of a person with no Rh activity agree with the hypothesis of an inhibitor gene acting on the biosynthetic pathway of Rh antigens.		
9. KEY WORDS Allergy, enzymes, immunology, antigen, antibody.		
10. SUPPORTING PROJECTS Not Applicable		
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet	12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet	
<div style="display: flex; justify-content: space-between;"> DA FORM 1309R 1 June 63 PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of ____ </div>		

ACCESSION NO.
36169

ARMY RESEARCH TASK REPORT

13. PROJECT, TASK OR
SUBTASK NUMBER

11 22 23 24 25 26 27 28 29
3 A 0 1 2 5 0 1 B 8 1 3 0 5 0 1

14. DATE OF REPORT (30-33)

30 33 34
0 6 6 4 4

15. SECURITY OF WORK (34)

16. TYPE OF REPORT

35 36 47 48 49 50 51 52 55
3 1 2 6 3

17. SCIENTIFIC FIELD
a. Topical Classific. (56-61)
b. Functional Class (62-64)

56 61 62 64
0 1 0 6 0 2

18. OSD CLASSIFICATION
(65-66)

65 66
BR

19. R&D CATEGORY (67)

67
1

20. CONTRACT NUMBER

11 12 13 14 15 17 18 21 22 26 27
D A

21. GRANT NUMBER

28 29 30 33 34 35 36 38 39 40 41 45 46
D A G

22. ESTIMATED COMPLET.
DATES

47 51 52 56 57 61 62 66 67 71
1 C O N T 2 3 4 5

23. PRIORITY (11-14)

24. PROGRAM ELEMENT
(15-26)

11 14 15 26
2 6 • 1 1 • 2 5 • 0 1 • 1

25. CMR&D CODES

27 29 30 32 33 35
N / A

26. CDOG REFERENCE

a. Paragraph No. (36-44)

b. Functional Group (45)

36 39 40 41 42 43 44 45
1 4 1 2 a 6

27. FUNDING

a. Est. Total Cost (11-15)

b. % Spent Intern. (16-18)

" " Extern. (19-21)

c. Total Obligation (22-26)

d. Progrmd. Cur. FY (27-33)

e. " " " +1 (34-40)

f. " " " +2 (41-47)

g. " " " +3 (48-54)

h. " " " +4 (55-61)

i. " " " +5 (62-68)

j. " " " +6 (69-75)

k. Total Man Years of
Effort (75-78)

11 15 16 18 19 21 22 26
1 2
27 28 29 33 34 35 36 40
41 42 43 47 48 49 50 54
55 56 57 61 62 63 64 68
69 70 71 75 76 78

DA FORM
1 June 63 1309R

Previous Editions are Obsolete

Page 2 of _____

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

- (P) Alexander, B.H., Ph.D., Dept of Immunochemistry
DCD&I, WRAIR, WRAMC, Washington, D.C. 20012
576-3780 or Interdepartmental Code 198, Ext 3780 49
- (P) Austen, K.F., M.D., Dept of Medicine
Massachusetts General Hospital
Boston, Massachusetts 19
- (P) Barbaro, J.F., Ph.D., Dept of Immunochemistry
DCD&I, WRAIR, WRAMC, Washington, D.C. 20012
576-3659 or Interdepartmental Code 198, Ext 3659 49
- (P) Basch, R., Capt, MC, Dept of Immunochemistry
DCD&I, WRAIR, WRAMC, Washington, D.C. 20012
576-3664 or Interdepartmental Code 198, Ext 3664 49
- (P) Boone, B., B.S., Dept of Biological Chemistry
Div. of Biochem., WRAIR, WRAMC, Washington, D.C. 20012
576-3527 or Interdepartmental Code 198, Ext 3527 49
- (P) Camp, F.R., Jr., Maj, MSC, Dept of Immunochemistry
DCD&I, WRAIR, WRAMC, Washington, D.C. 20012
576-3760 or Interdepartmental Code 198, Ext 3760 49
- (P) Christian, G., Ph.D., Dept of Clinical Chemistry
Div. of Biochem., WRAIR, WRAMC, Washington, D.C. 20012
576-3527 or Interdepartmental Code 198, Ext 3527 49
- (P) Gibbs, M.B., M.S., Dept of Immunochemistry
DCD&I, WRAIR, WRAMC, Washington, D.C. 20012
576-3760 or Interdepartmental Code 198, Ext 3760 49
- (P) Hafner, L., M.S., Dept of Immunochemistry
DCD&I, WRAIR, WRAMC, Washington, D.C. 20012
576-3780 or Interdepartmental Code 198, Ext 3780 49
- (P) Hayden, A.H., M.S., Dept of Immunochemistry
DCD&I, WRAIR, WRAMC, Washington, D.C. 20012
576-3458 or Interdepartmental Code 198, Ext 3458 49
- (P) McConnell, D., Capt, MC, Dept of Immunochemistry
DCD&I, WRAIR, WRAMC, Washington, D.C. 20012
576-3664 or Interdepartmental Code 198, Ext 3664 49
- (P) Schur, P., Capt, MC, Dept of Immunochemistry
DCD&I, WRAIR, WRAMC, Washington, D.C. 20012
576-3659 or Interdepartmental Code 198, Ext 3659 49

Barbaro, J.F. Demonstration of a Haemolytically Active 11S Component of Rabbit, Guinea Pig and Human Serum by Means of Antigen-Antibody Precipitates. Nature 199:819 (1963)

Alexander, B.H., Hafner, L.S., Garrison, M.V. and Brown, J.E. Another Example of the Novel Conversion of a Phosphonate to a Phosphate J.Org.Chem. 28:3499 (1963)

Camp, Frank R. Jr. Blood Banking: Retrospection of People, Problems and Progress Gained from a Decade of Experiences in overseas Military Operation. Military Medicine 128:1222 (1963)

ANNUAL PROGRESS REPORT

Project No. 3A012501B813

Title: Army Medical Basic Research
in Life Sciences

Task No. 05

Title: Immunology

Subtask No. 01

Title: Antigen-antibody reactions
in vivo and in vitro

Description: The purpose of this task is to study the phenomena involved in the agglutination reaction of the human blood group system, the enzymatic mechanisms of allergic reactions, and the quantitation of gel precipitin reactions.

Progress:

1. Phosphonate ester inhibition of guinea pig C'1a

a. Several series of p nitrophenyl ethyl R phosphonates, in which R equals phenylalkyl, alkyl and -chloro-alkyl and -amino alkyl, have been used to characterize guinea pig activated first component of complement, C'1a, by the pattern of inhibition they give with this enzyme. The inhibitors and enzyme were reacted at pH 8.0, and 25° so that the results would be comparable to those obtained in previous studies with acetyl cholinesterase, trypsin and chymotrypsin.

b. In the phenylalkyl series, C'1a was found to differ from both in having maximum reactivity with the benzylphosphonate, but was similar to trypsin in showing minimal reactivity with the phenylethyl phosphonate. In the alkyl series, C'1a was maximally inhibited by the butyl phosphonate, whereas, trypsin and chymotrypsin were optimally inhibited by the hexyl and heptylphosphonates, respectively. In the chloro-alkyl series, both C'1a and trypsin showed a peak in activity with the 3-chloropropylphosphonate, however, C'1a gave minimum in activity with the 5 chloropentylphosphonate, whereas, with trypsin the minimal activity was present with the 6-chlorohexylphosphonate. Chymotrypsin yielded a single peak in reactivity with the 6-chlorohexylphosphonate. The 5 amino-pentyl and 6 amino hexylphosphonates had an hundred fold increased inhibitory activity against C'1a compared to the corresponding alkylphosphonates; against trypsin the increase in inhibitory activity was 4000 and 800 respectively, whereas, with chymotrypsin the amino alkyl compounds were actually less inhibitory than the corresponding alkyl phosphonates.

c. It was concluded that C'1a and trypsin were more similar than chymotrypsin in the specificity of their reactions with phosphonate esters, and on this basis, C'1a and trypsin were classified as "parazymes" i.e. enzymes which are similar but not identical in specificity. The

basis for the similarity is believed to be the possession in both enzymes of a negatively charged binding site at some definite distance from the esteratic site.

2. Phosphonate Inhibition of Antigen Induced Histamine Release From Sliced Guinea Pig Lung.

a. The same phosphonates used above were also employed as inhibitors of histamine release from sliced perfused lung upon the addition of antigen (egg albumin). The lung slices were obtained from guinea pigs actively sensitized to egg albumin. A straight line relationship was obtained when the logarithm of the histamine released as percentage of the histamine released in the control uninhibited samples was plotted against the concentration of inhibitor. From these results it was inferred that the amount of histamine released was proportional to the degree of activation by the antigen of the organophosphorus inhibitable enzyme present in the lung slices.

b. In the phenylalkyl series a maximum inhibition of histamine release was given by the benzylphosphonate. In the alkyl series, the maximum came with the butylphosphonate. In the chloro-alkyl series, the 3 chloropropyl and 4 chlorobutyl phosphonate were equal in inhibitory power, and gave the greatest inhibition. There was no minimum in inhibition noted. The amino alkylphosphonates gave less inhibition of histamine release than the corresponding alkylphosphonates.

c. It was tentatively concluded that the C'1a and the antigen activated organophosphorus inhibitable esterase in guinea pig lung are similar in the pattern of specificity of their inhibition by phosphonate esters but not identical. This conclusion must remain tentative until questions concerning the differential access of the inhibitors to the target cell in the guinea pig lung are answered.

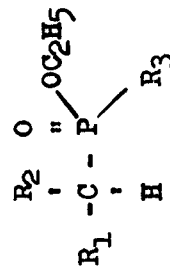
3. The Synthesis of Phosphonate Esters

The preparation of new phosphonates inhibitors is continued. Thirty six hitherto unknown and unreported compounds are recorded in Table I, and Table II.

4. Disulfide bond analysis of Gamma Globulin

A method was developed for the amperometric titration of inter and intra-chain disulfide bonds of gamma globulin. The total disulfide bonds were measured by reducing the gamma globulin in 8 M guanidine with 0.5 M Na_2SO_3 titrating with mercuric chloride. The inter-chain disulfide bonds were measured by reducing the gamma globulin with 0.5 M Na_2SO_3 in the absence of guanidine, and titrating with phenylmercuric hydroxide at 0°. The results are given in Table ~~II~~

Table III



Empirical Formula	R ₁	R ₂ $\frac{\text{O}}{\text{O}}$	R ₃	B.P. (°C)	mm	n _D ²⁵	T	C	H	T	C	P
1. C ₁₅ H ₂₃ O ₅ P	C ₆ H ₅ C ₂ H ₄	CH ₃ CO	OC ₂ H ₅	144-8	0.25	1.4830	57.3	56.5	7.4	7.4	9.9	9.3
2. C ₁₄ H ₂₁ O ₅ P	C ₆ H ₅ CH ₂	$\frac{\text{O}}{\text{CH}_3\text{CO}}$	OC ₂ H ₅	118-20	0.3	1.4891	56.0	56.4	7.1	7.2	10.3	10.3
3. C ₆ H ₁₄ ClO ₃ P	CH ₂ Cl	H	OC ₂ H ₅	120	20.0	1.4405	--	--	-	-	-	-
4. C ₁₃ H ₁₉ O ₄ P	C ₆ H ₅ C ₂ H ₄	=O	OC ₂ H ₅	137-40	0.03	1.4938	57.8	57.6	7.1	7.1	-	-
5. C ₁₂ H ₂₅ O ₅ P	C ₅ H ₁₁	$\frac{\text{O}}{\text{CH}_3\text{CO}}$	OC ₂ H ₅	97-100	0.3	1.4290	51.4	51.1	9.0	8.9	11.1	11.2
6. C ₁₄ H ₂₉ O ₅ P	C ₇ H ₁₅	$\frac{\text{O}}{\text{CH}_3\text{CO}}$	OC ₂ H ₅	117	0.1	1.4365	54.5	54.2	9.5	9.6	10.1	9.9
7. C ₁₃ H ₂₇ O ₅ P	C ₆ H ₁₃	$\frac{\text{O}}{\text{CH}_3\text{CO}}$	OC ₂ H ₅	124	0.3	1.4346	53.1	53.0	9.2	9.7	10.5	10.5
8. C ₁₂ H ₁₉ O ₄ P	C ₆ H ₅	OCH ₃	OC ₂ H ₅	128	0.4	1.4920	55.8	55.7	7.4	7.5	12.0	11.8
9. C ₁₅ H ₂₅ O ₆ PS	C ₃ H ₇	$\frac{\text{O}}{\text{CH}_3\text{C}_6\text{H}_4\text{SO}}$	OC ₂ H ₅	110	A	1.4979	49.5	50.2	6.9	7.5	8.5	8.5
10. C ₁₈ H ₂₁ O ₅ P	C ₆ H ₅	$\frac{\text{O}}{\text{C}_6\text{H}_5\text{CO}}$	OC ₂ H ₅	134	A	1.5375	62.1	62.5	6.1	6.1	8.9	8.6

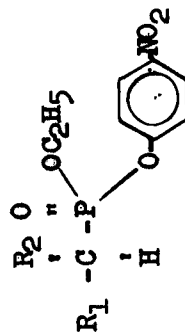
Table I (Continued)

Empirical Formula	R ₁	R ₂	R ₃	B.P. (°C)	mm	n _D ²⁵	C	H	P
							T	T	T
							C	C	C
11. C ₁₀ H ₂₃ O ₄ P	C ₄ H ₉	OCH ₃	OC ₂ H ₅	132	12	1.4266	50.4	50.3	13.0
12. C ₈ H ₁₇ O ₅ P	C ₃ H ₇	CH ₃ CO	OH	110	A	1.4406	42.8	42.4	13.8
13. C ₁₅ H ₁₅ O ₄ P	C ₆ H ₅	=O	OC ₆ H ₅	B	B	--	62.1	61.0	10.7
14. C ₁₆ H ₂₀ NO ₃ P	C ₆ H ₅	CH ₃ N	OC ₆ H ₅	134	A	1.5390	62.9	63.2	10.1
15. C ₈ H ₁₈ ClO ₃ P	C ₄ H ₉	OCH ₃	Cl	73	0.1	1.4357	--	--	--
16. C ₉ H ₁₈ ClO ₄ P	C ₄ H ₉	CH ₃ CO	Cl	95	0.1	1.4446	--	--	--
17. C ₁₁ H ₂₂ ClO ₄ P	C ₆ H ₁₃	CH ₃ CO	Cl	110	A	1.4445	--	--	--
18. C ₁₂ H ₂₄ ClO ₄ P	C ₇ H ₁₅	CH ₃ CO	Cl	110	A	1.4451	--	--	--
19. C ₁₂ H ₁₆ ClO ₄ P	C ₆ H ₅ CH ₂	CH ₃ CO	Cl	110	A	1.5066	--	--	--
20. C ₁₀ H ₁₆ ClO ₄ P	C ₅ H ₁₁	CH ₃ CO	Cl	94-5	0.3	1.4454	--	--	--
21. C ₄ H ₉ ClO ₂ P	CH ₂ Cl	H	Cl	115	20.0	1.4698	--	--	--

A Molecular still

B m.p. 68-9°

Table II



Empirical Formula	R ₁	R ₂	B.P.* (°C)	n_D^{25}	C	H	N	P
					T	F	T	F
1. C ₁₄ H ₂₀ NO ₇ P	C ₃ H ₇	CH ₃ CO	110	1.5059	48.7	48.6	4.1	3.6
2. C ₁₅ H ₂₂ NO ₇ P	C ₄ H ₉	CH ₃ CO	135	1.5019	50.1	50.3	3.9	4.2
3. C ₁₆ H ₂₄ NO ₇ P	C ₅ H ₁₁	CH ₃ CO	154	--	51.5	51.7	3.8	3.8
4. C ₁₇ H ₂₆ NO ₇ P	C ₆ H ₁₃	CH ₃ CO	154	1.4997	52.8	52.6	3.6	3.3
5. C ₁₈ H ₂₈ NO ₇ P	C ₇ H ₁₅	CH ₃ CO	154	1.4976	53.9	53.9	3.5	3.3
6. C ₁₈ H ₂₀ NO ₇ P	C ₆ H ₅ CH ₂	CH ₃ CO	184	1.5434	55.0	55.1	3.6	3.5
7. C ₁₉ H ₂₂ NO ₇ P	C ₆ H ₅ C ₂ H ₄	CH ₃ CO	154	1.5391	56.0	56.5	3.4	3.4
8. C ₁₀ H ₁₃ ClNO ₅ P	CH ₂ Cl	H	**	--	40.9	41.0	4.8	4.9
9. C ₂₁ H ₂₂ NO ₇ P	C ₆ H ₅	C ₆ H ₅ CO	135	1.5498	59.9	59.5	3.2	2.5

* Molecular still

** m.p. 55-60

Table II (Continued)

Empirical Formula	R ₁	R ₂	B.P.* (°C)	n _D ^{25°}	C		H		N		P	
					T	F	T	F	T	F	T	F
10. C ₁₄ H ₂₂ NO ₆ P	C ₄ H ₉	OCH ₃	135	1.5138	50.7	50.6	6.7	7.1	4.2	4.6	9.3	8.6
11. C ₁₃ H ₂₀ NO ₆ P	C ₃ H ₇	OCH ₃	135	1.5172	49.2	48.7	6.4	6.6	4.4	4.4	-	-
12. C ₁₆ H ₂₆ NO ₆ P	C ₆ H ₁₃	OCH ₃	135	1.5034	53.5	54.1	7.3	7.6	-	-	8.6	8.4
13. C ₁₆ H ₁₈ NO ₆ P	C ₆ H ₅	OCH ₃	135	1.5572	54.7	54.7	5.2	5.1	4.0	3.6	8.8	8.6
14. C ₁₂ H ₁₆ NO ₆ P	C ₃ H ₇	=O	135	1.5211	47.8	48.3	5.4	5.3	4.7	4.6	-	-
15. C ₁₄ H ₂₀ NO ₆ P	C ₅ H ₁₁	=O	135	1.5130	51.1	49.7	6.1	6.1	4.2	4.0	9.4	8.1

*Molecular still

Table III

	Total Disulfide	Inter-chain Disulfide
7S Rabbit gamma globulin	22.5 \pm 0.5	3
5S Rabbit gamma globulin	17	3
7S Sheep gamma globulin	22	-
5S Sheep gamma globulin	15	-
7S Human gamma globulin	18	-

5. Role of disulfide bonds in complement fixing and precipitating properties of 7S rabbit and sheep antibodies.

The 7S antibody containing sheep and rabbit gamma globulins were reduced with increasing concentrations of 2-mercapto-ethanol and alkylated with iodoacetamide. The number of disulfide bonds remaining was measured, and the complement fixing and precipitating ability were studied quantitatively. The reduction of 7 disulfide bonds in both preparations was associated with a 90 percent decrease in complement fixing efficiency but had no effect on the precipitating ability. The reduction of 10-11 disulfide bonds was associated with no further decrease in complement fixing activity, but there was a distinct decrease in precipitating activity.

6. The complement fixing ability of sheep antiserum on 7S sheep gamma globulin

Preformed immune aggregates from sheep antiserum or from 7S gamma globulin fix more complement at 37° than immune aggregates formed in the presence of complement. Immune aggregates formed from 7S sheep antibody in the presence of complement fixes more complement at 4°C than preformed immune aggregates. The non-immune gamma globulin appears to competitively inhibit complement fixation by antibody and antigen much more at 37° than at 4°C.

7. Automatic intermittent flow dialysis

In cooperation with the Instrumentation Division, WRAIR, a completely automated intermittent flow dialysis apparatus has been developed. This electronically controlled apparatus permits fluid to be changed every 4 hours throughout the night or over a weekend.

8. The site and mechanism of inhibition of complement by aromatic amino acid derivatives.

Cushman, Becker, and Wirtz, J. Immunol. 79:80, 1957 had previously demonstrated that aromatic amino acid derivatives gave inhibition of guinea pig complement. Present work has succeeded in demonstrating that the primary site of action of the inhibitors is in the reaction of EAC'1a,4,2a with C'3. More specifically, the site has been located in the formation of the heat stable intermediate (EAC'1a,4,2a,3cb in the terminology of Linscott and Nishikawa) by EAC'1a,4,2a,3c (the heat labile intermediate capable of giving immune adherence). Immune adherence is also inhibited by the aromatic amino acid derivatives; the rank order of inhibition of these compounds is approximately the same as their rank order of inhibition of the EAC'1a,4,2a and C'3 reaction. Kinetic evidence was obtained that the inhibition of the formation of the heat labile intermediate by acetyl L tyrosine was competitive.

9. Antigen induced histamine release

a. Preformed antigen-antibody precipitates treated with normal rabbit plasma in the presence of 0.01 M EDTA are capable of removing the 11S component of hemolytic complement while C'1 activity is fully retained; the ability to sustain histamine release from rabbit platelets on the addition of well washed immune precipitates was also lost. The addition of 11S component prepared from human serum by the technique of Muller-Eberhard fully restored hemolytic activity; however at no concentration of 11S was more than 50% of the histamine releasing ability restored.

b. Isolated rabbit and sheep gamma globulin containing antibody to human serum albumin were treated with either pepsin to form 5S gamma globulin, or varying concentrations of the reducing agent mercapto-ethylamine. Immune precipitates were prepared from the gamma globulin before and after treatment. The various treatments did not affect the histamine releasing ability of the precipitates; 80-120 ug of precipitate nitrogen was required for maximal histamine release in all cases. However the complement fixing ability of 5S precipitates from both rabbit and sheep gamma globulin was greatly decreased, requiring 80-200 times more antibody than the untreated 7S globulin to fix 50% of the complement. Treatment of 7S globulin with mercapto-ethylamine reduced the complement fixing ability of the rabbit and sheep precipitates by 2/3 but had no effect on the histamine releasing capacity.

10. Heparin inhibition of the kinin forming system.

A concentration of 500 units of heparin/ml of human blood prevents the activation of the kinin forming system in plasma, but not in serum. The site of inhibition probably involves the early stages of clotting which are shared with the kinin forming system. Fifty percent inhibition of Pf/dil occurs at 320 units of heparin/ml of reaction mixture;

fifty percent inhibition of kallikrein occurs at 1950 units heparin per ml. This finding provides a quick, simple method of differentiating between these two permeability globulins.

11. Antigen-antibody reactions in agar.

a. The major factor standing in the way of routine use of the quantitative gel precipitin technique described previously is the lack of a suitable, not too expensive densitometer. The only ones found suitable previously have cost \$4500 - \$5500. Recently, the Aminco Co. at our urging has designed an attachment to their photomultiplier microphotometer suitable for reading the precipitate density of Oudin tubes; the instrument with attachment will be much less expensive. It has been thoroughly checked in our laboratory and found quite suitable.

b. Oudin experiments were done using rabbit anti-human serum albumin, and phosphate buffer and fresh normal rabbit serum as diluents. Antiserum diluted with unheated normal serum and then added to agar under circumstances where the temperature never went above 33°C gave distinctly higher precipitate optical density than antiserum diluted with phosphate buffer. Antiserum diluted with normal serum which had been heated at 53° for 20 minutes, and then Oudin tubes prepared at 33°C give the same optical density as when fresh, unheated normal serum was used. Antiserum diluted with normal serum heated to 53°, and processed at 53° instead of 33° gave an optical density in Oudin tubes of 63% of the optical density of antiserum and buffer processed at either 33° or 53°. Although no effect was seen of heating normal serum diluent at 53° when the Oudin tubes were prepared at 33°, if the diluent was heated at 63° for 20 minutes, a 60 percent fall in optical density was noted even when the normal serum and antiserum were processed at 33°.

c. There is general agreement that in liquid medium more chicken antibody is brought down in 1.5 M salt than in the more usual 0.15 M. However, using chicken antiserum in Ouchterlony reactions different investigators have come to contradictory conclusions as to the effect of salt concentration. Investigation of this problem has established that almost all antisera obtained within the first month after injection tested in Ouchterlony plates at high salt gave denser precipitate lines than when tested at low salt. Conversely, all antiserum obtained 3 months after initial injection gave denser precipitate lines when tested in low salt.

12. Application of an electronic free cell counting technic to quantitative studies in hemagglutination.

It would be possible to modify the Coulter electronic cell counter to provide free cell counts of undiluted test mixtures if a simple means of selecting the upper threshold were found by which the instrument would automatically adjust the threshold. The method of measuring agglutination with upper thresholds at the modal peak of red cell size distribution curves reported by Bowdler and Swisher was investigated for this

purpose. Their findings could not be substantiated that instrument assay curves obtained in this manner are comparable to hemocytometer curves since the instrument assay curves have significantly lower slopes.

13. Studies of the reactivities of iso-agglutinins with A, and B red cell antigens

a. It has been reported from this laboratory that A₁ antigens fall into three quantitative categories of 100%, 75% and 40% relative strength, and B antigens into categories of 100%, 60% and 40% relative strengths. Investigations still under way indicate that these relative activities of A₁ and B antigen depend on the antiserum employed. Only three of seven commercial anti-A sera and one of seven sera from individuals immunized with hog and human group A substances gave the original pattern of relative reactivities. With most antisera only two subgroups could be differentiated; the two strongest antigens were indistinguishable. With one serum from a group O individual immunized with group O cells, the relative strengths were 100%, 90% and 75% respectively, whereas, another similar serum did not differentiate the two stronger antigens and assigned a relative strength of 40% to the weakest antigen.

b. Only three of nine anti-B sera gave the standard picture when tested with two B cells of the 100% and two of the 60% category. With the other six anti-B sera cells the 4 cells were differentiated in to 100%, 80%, 60%, and 40% relative activity.

14. Population study of the ABO antigens of a tribe of South American Indians.

In collaboration with the Department of Hematology, WRAIR, the ABO blood types of 302 members of a tribe from the upper reaches of the Amazon were tested. The frequency of group O is 96%, group A, 2.9%, and group B is 1.1%.

15. Thermodynamic studies of the B-anti B system

a. In these studies a weak B cell was selected as the standard test cell. Antisera were absorbed with concentrations of B cells varying from 3000 to 25,000 mostly at room temperature, but also in some experiments at 37° and 4°. In most experiments the supernatants were tested for hemagglutinating activity at room temperature, but also at 37° and 4°.

b. When as suggested by Klotz the reciprocal of the antibody activity fixed per cell was plotted against the reciprocal of the antibody free a straight line was obtained. The ratios of the slopes of the straight lines obtained by carrying out the absorptions at 25° and 37° are different from different ABO genotypes in confirmation of the findings of the Wurmser group. However, studies of supernates absorbed at 4°

showed such variations in the slope of the lines that interpretation of these results without additional information is impossible at this time.

c. Using the method of plotting suggested by Scatchard, in which the ratio of the antibody fixed per cell to the antibody free is plotted against the antibody fixed per cell quite anomalous results were obtained. The slope and configuration of the curve depended on the concentration of cells used in absorption. This indicates strongly that the binding affinities of antiB antibodies from apparently unstimulated individuals are not homogeneous, in contradiction to the conclusion of the Wurmsers.

16. Quantitative Studies of the A_x antigens

The studies of the A_x antigens of numerous families conducted in collaboration with Dr. Frank Ellis of the Wayne County General Hospital, Michigan, were completed. Bloods from individuals previously reported to be of weak A subgroups A_4 , A_0 and A_m were shown to give parallel log probit assay curves with slopes significantly lower than those of A_2 cells. The strengths of these weak A antigens differed as: A_4 A_0 A_m . These findings provide experimental evidence in support of the recent suggestion of Race and Sanger that weak A antigens would not be differentiated but classified as a single group, designated A_x . The A_x antigens of eight families fell into 6 quantitative subgroups. The finding that the A_x antigens of the two siblings and their offspring were of identical strengths supports the hypothesis that A_x is an expression of modifier genes operating on A_2 or weak A_1 antigens, however, could not be excluded since the A_x antigen of a mother was 50% weaker than that of her 10 year old child. The finding that the lack of equilibrium seen with A_1 and B cells is also observed with A_x cells discredits the theory of Wiener that cross-reactive antibodies of group O sera have anti-C specificity and react with a C antigen on A and B cells.

17. Quantitative studies of the Rh antigens of a family postulated to possess an Rh inhibitor gene.

In collaboration with Dr. Philip Levine of the Ortho Research Foundation studies of the Rh antigens of members of a family postulated to possess an inhibitor gene acting on the biosynthetic pathway of Rh antigens were initiated. The red cells of the propositus exhibited no Rh activity (---/---) but she transmitted CDe complex to her child whose genotype is CDe/cde. Quantitative assays of the C, D, c, and e antigens of the parents, two siblings, four paternal and one maternal relatives revealed decreased activities of all Rh antigens from that observed with standard cells of the same genotypes. The exception to this was one paternal uncle whose C, D, and e antigens were comparable in strengths or stronger than the antigens of the standard cells. These findings suggest inhibitor gene action in these individuals, however, it is not known whether normal Rh complexes exist in the population with similar weak Rh antigens. It was also found that the Rh antigens of the child of the propositus were as weak as those of his maternal grandmother. The fact that the homozygous c antigens of the father of the child

(cde/cde genotype) were as strong as those of the cde/cde standard cell, whereas the single c antigen of his child possessed only 16% of the strength of the single c antigen of three R₁r standard cells provides suggestive evidence that an incompletely recessive or semi-dominant inhibitor gene on a chromosome other than the Rh chromosome has suppressed expression of Rh antigens in the homozygous propositus and greatly reduced the Rh activities of eight out of nine heterozygous members of this family. Future studies of the nine siblings of the mother of the propositus should provide more definitive evidence of inhibitor gene action in the heterozygote. This mode of gene action has not been described for human blood group systems.

18. Studies of the interaction of the Rh-Er antigen as evidenced by the phenotypic expression of their red cell antigens.

The collaborative project with Dr. Richard Rosenfield of the Mt. Sinai Hospital, New York City, to confirm previous observations that the Cde gene inhibits expression of the D antigen in a manner predictable by the sensitive log-probit assay method was completed. The results of the study of the C,D,E, and c antigens of the bloods from the parents and 13 off-spring of a family possessing the CDe, cde, cD^uE and Cde complexes in various combinations showed that dosage could be demonstrated for the D,E, and c antigens but not the C antigen. There was no demonstrable interaction of D antigen on the expression of C, c or E since antigens partnered with the weak D of the cD^uE complex in trans position showed equal activities as those partnered with the strong D of the cDE complex. The finding that the E activity of the cde/cde is the same as that of the CDe/cde does not accord with the report that D had a depressing effect on E in cis position. The quantitative inhibition of D antigen of the CDe complex by the Cde in trans position found in our previous family study was confirmed. An inhibitory action of C on e and E antigens was also observed, the effect being greater when the C is in the Cde than CDe complex. A single c antigen expresses 47% the activity of homozygous cells when partnered with the CDe complex and only 2.3% of this activity when the companion complex is Cde. The single E antigen expresses only 50% the activity of homozygous cells when in trans position to the CDe and only 10% this activity with the Cde complex. These observations have not been reported heretofore.

19. Evaluation of a manifold washing process for preparing erythrocytes for the anti-human globulin (Coombs) test.

In order to eliminate the tedium associated with washing large numbers of samples of red cells for the Coombs test, a simple manifold device was designed which would permit simultaneous aspiration and dispensation of wash solutions into twelve tubes without removing the tubes from the centrifuge. The efficiency and effectiveness of the manifold washing process was tested by comparing results of direct and indirect Coombs tests employing manifold and manual washed erythrocytes. Comparable results were found with both methods, however, a weak Kell antigen was detected only with manifold washed red cells and in many instances titers of various incomplete antisera were one tube higher with manifold washed red cells.

Summary and Conclusions:

1. Several series of p nitrophenyl ethyl phosphonates have been used to study the inhibition of guinea pig activated first component of complement, C'1a. C'1a was found to be more similar to trypsin than to chymotrypsin and acetylcholinesterase in its pattern of inhibition.
2. These same phosphonates were used to study the inhibition of antigen-induced histamine release from sliced guinea pig lung tissue. The pattern of inhibition was similar but not identical to that of C'1a.
3. Synthesis of phosphate esters is continued. Thirty-six hitherto unknown and unreported compounds were synthesized.
4. A method for the amperometric titration of inter and intra-chain disulfide bonds has been developed, and applied to the study of the disulfide bonds of gamma globulin.
5. The reduction of disulfide bonds has been quantitatively correlated with the fall in complement fixing and precipitating ability of rabbit and sheep 7S antibody.
6. Non-immune gamma globulin appears to inhibit complement fixation by immune aggregates more at 37° than at 4° C.
7. In cooperation with the Instrumentation Division, WRAIR, a completely automatic intermittent flow dialysis apparatus has been developed.
8. Aromatic amino acid derivatives competitively inhibit the formation of the heat stable intermediate (EAC'1a,4,2a,3cb) from both EAC'1a,4,2a and EAC'1a,4,2a,3c.
9. Human 11S can restore hemolytic activity to an R11S but not its ability to support histamine release. Pepsin as well as treatment of sheep and rabbit antibody with reducing agents have no discernible effect on their ability to effect histamine release from rabbit plasma and platelets.
10. Heparin in high concentration prevents activation of permeability globulins in human plasma but not in human serum. Kallikrein is much more resistant to the inhibitory activity of heparin than is Pf/dil.
11. Chicken antisera from early bleedings gave denser bands in Ouchterlony tests with 1.5 M NaCl than with 0.15 M NaCl; the reverse was true of later bleedings.
12. Application of an electronic free cell counting technique to quantitative hemagglutination assay was studied.
13. The classification of the B and A₁ cells into quantitative categories depends on the antiserum used.

14. In collaboration with the Department of Hematology, WRAIR, a population study of the ABO antigens of a tribe of South American Indians was undertaken.

15. Thermodynamic studies of the B-anti-B system reveal a heterogeneity of binding affinities of the anti-B antibody present in the sera of presumably unstimulated individuals.

16. Quantitative studies of the A_x antigen support the hypothesis of quantitative inheritance of an A_x gene, but do not support the hypothesis of Wiener that cross-reactive antibodies of group O sera have anti C activity.

17. Family studies of the red cells of an individual possessing no Rh activity are compatible with the hypothesis of an inhibitor gene acting on the biosynthetic pathway of Rh antigens.

18. Confirmation was obtained of previous findings that the CDe gene inhibits expression of the D antigen in a manner predictable by the sensitive log probit hemagglutination assay method.

19. A simple manifold washing process for preparing erythrocytes for anti-human globulin (Coombs) test was designed and evaluated with the manual washing procedure.

ARMY RESEARCH TASK REPORT				REPORTS CONTROL SYMBOL CSCRD-6(R2)	
ACCESSION NUMBER 36170			PROJECT, TASK, OR SUBTASK NO. 3A012501B8130502		
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D. C., 20315			2. FUNDING AGENCY Army Medical R&D Command Office of The Surgeon General Washington, D. C., 20315		
3. CONTRACTING AGENCY NA			4. CONTRACTOR AND/OR GOV'T LABORATORY A Walter Reed Army Inst of Rsch Walter Reed Army Medical Center Washington, D. C., 20012 723-1000, Ext 3552		
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Randall, Raymond, D. V. M., Dept of Hazardous Operations Div of Comm Dis & Imm, WRAIR, WRAMC, Washington, D. C., 20012 576-5109 or Interdepartmental Code 198, Ext 5109. See Contin. Sheet					
6. TITLE OF: PROJECT <input type="checkbox"/> TASK <input type="checkbox"/> SUBTASK <input checked="" type="checkbox"/> Immunization studies of exotic diseases. (U)					
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964					
8. RESUME (U) Chikungunya is a serious though rarely fatal virus disease of man widely disseminated through Africa and Asia. The purpose of this investigation is to determine the most suitable agent within the Chikungunya complex which lends itself to the production of a vaccine offering broad spectrum protection in human beings. Rhesus monkeys have been subjected to multiple challenge procedures with the following Group A arboviruses: CHIK-168, CHIK-E103, BAH-306, Onyong-nyong, Mayaro B and Venezuelan equine encephalomyelitis. The serologic and immunologic patterns observed indicated that a broad protective inter-relationship exists between African and Asian Chikungunya and to a lesser degree with Mayaro B. Pre- and post-challenge sera were assayed by hemagglutination-inhibition, complement-fixation and serum neutralization techniques. Extensive serologic overlap was characteristic of the agents within this group. An experimental vaccine was prepared with CHIK-168 and assayed in mice. The excellent results obtained indicated that a vaccine trial in monkeys would be feasible. This phase is currently being carried out. During the current report period 1400 doses of Rift Valley fever (RVF) vaccine have been supplied to requesting agencies. Neutralization tests are being done on serum samples obtained from the vaccinees.					
9. KEY WORDS Chikungunya, serologic and immunogenic patterns, vaccine production and assay, Rift Valley fever, vaccine for human use.					
10. SUPPORTING PROJECTS Not applicable.					
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet			12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet		
FORM 1309R DA 1 June 63 PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of ____					

ACCESSION NO.
36170

ARMY RESEARCH TASK REPORT

13. PROJECT, TASK OR
SUBTASK NUMBER

11 22 23 24 25 26 27 28 29
3 A 0 1 2 5 0 1 B 8 1 3 0 5 0 2 1 2 6 3

14. DATE OF REPORT (30-33)

30 33 34
0 6 6 4 4

15. SECURITY OF WORK (34)

16. TYPE OF REPORT

35 36 47 48 49 50 51 52 55
3 1 2 6 3

17. SCIENTIFIC FIELD

- a. Topical Classific. (56-61)
b. Functional Class (62-64)

56 61 62 64
0 1 0 6 0 2 1

18. OSD CLASSIFICATION
(65-66)

65 66 67
B R 1

19. R&D CATEGORY (67)

20. CONTRACT NUMBER

11 12 13 14 15 17 18 21 22 26 27
D A 1 1 1 1 1 1 1 1 1 1

21. GRANT NUMBER

28 29 30 33 34 35 36 38 39 40 41 45 46
D A 1 1 1 1 1 1 1 1 1 1 1 1

22. ESTIMATED COMPLET.
DATES

47 51 52 56 57 61 62 66 67 71
1 C O N T 2 3 4 5

23. PRIORITY (11-14)

24. PROGRAM ELEMENT
(15-26)

11 14 15 26
2 6 1 1 2 5 0 1 1

25. CMR&D CODES

27 29 30 32 33 35
N / A 1 1 1 1

26. CDOG REFERENCE

- a. Paragraph No. (36-44)
b. Functional Group (45)

36 39 40 41 42 43 44 45
1 4 1 2 1 a 6

27. FUNDING

- a. Est. Total Cost (11-15)
b. % Spent Intern. (16-18)
" " Extern. (19-21)
c. Total Obligation (22-26)
d. Progmd. Cur. FY (27-33)
e. " " " +1 (34-40)
f. " " " +2 (41-47)
g. " " " +3 (48-54)
h. " " " +4 (55-61)
i. " " " +5 (62-68)
j. " " " +6 (69-75)
k. Total Man Years of
Effort (76-78)

11 15 16 18 19 21 22 26
1 2 1 2 1 2 1 2
27 28 29 33 34 35 36 40
1 1 1 1 1 1 1 1
41 42 43 47 48 49 50 54
1 1 1 1 1 1 1 1
55 56 57 61 62 63 64 68
1 1 1 1 1 1 1 1
69 70 71 75 76 78
1 1 1 1 1 1

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

(A) Binn, Leonard N., PhD., Dept of Hazardous Operations
Div Com Dis & Imm, WRAIR, WRAMC, Washington, D. C., 20012
576-5109 or Interdepartmental Code 198, Ext 5109 49

(A) Harrison, Venton R., M. S., Dept of Hazardous Operations
Div Com Dis & Imm, WRAIR, WRAMC, Washington, D. C., 20012
576-5109 or Interdepartmental Code 198, Ext 5109 49

REPORTS. Annual Progress Report, Walter Reed Army Institute of
Research, 1 July 1963 - 30 June 1964.

Binn, L. N., Randall, R., Harrison, V. R., Gibbs, C. J., and
Aulisio, C. G.: The Serological Reactions in a Case of Rift
Valley Fever. Am. J. Trop. Med. Hyg. 12:236, 1963.

Binn, L. N., Randall, R., Harrison, V. R., Gibbs, C. J., and
Aulisio, C. G.: Immunization Against Rift Valley Fever; the
Development of Vaccines from Non-primate Cell Cultures. Am.
J. Hyg. 77:160, 1963.

Randall, R., Binn, L. N., and Harrison, V. R.: Rift Valley
Fever Vaccine. Am. J. Trop. Med. Hyg., Vol. 12, 1963.

ANNUAL PROGRESS REPORT

Project No. 3A012501B813 Title: Army Medical Basic Research in Life Sciences
Task No. 05 Title: Immunology
Subtask No. 02 Title: Immunization studies of exotic diseases.

Description: This task is concerned with the development, production and evaluation of either live attenuated or formalin-killed vaccines against selected exotic diseases.

Progress:

1. Chikungunya and Related Viruses.

During the current report period work has been directed primarily toward the determination of serologic and immunologic overlap occurring between Chikungunya and other closely related Group A arboviruses, our objective being the production of an effective vaccine for human use against agents of the Chikungunya complex. These viruses are widely disseminated throughout Africa and Southeast Asia, and cause a serious, though rarely fatal, illness in man.

Since the last report period five additional groups of Rhesus monkeys were used for the purpose of studying the serologic and immunologic patterns produced by single and multiple challenge techniques with homologous and heterologous strains of the Chikungunya complex. The following agents have been employed for this study:

Chikungunya, strains 168 & E103 (African) and BAH-306 (Asian)
Mayaro B, strain 81 (South American)
Onyong-nyong, strain MP-87
Venezuelan encephalomyelitis (attenuated strain)

Because these viruses have an extremely limited host range, only suckling mice and tissue cultures have been employed for the determination of viremia in the monkeys, and gross clinical observations for overt signs of illness, elevation of temperature, and antibody response, have been used as criteria of infection.

Following the challenge techniques mentioned above, the monkeys within each group were bled and temperatured for five consecutive days for viremia and hematological determinations. Approximately 15 and 30 days post-challenge additional bleedings were made on each monkey for serologic and immunologic assays. These assays comprised hemagglutina-

tion-inhibition (HI), complement-fixation (CF) and serum neutralization (NA) tests. The serological tests were made with antigens prepared in suckling mouse brains with the several viral agents under study.

Serum neutralization tests were done employing the Porterfield technique of plaque inhibition in chick fibroblast tissue culture dishes. The Petri dishes were seeded with chick embryo fibroblasts, and, on the following day, inoculated with an appropriate dilution of the virus to give confluency (complete lysis). The tissue culture plates were then overlaid with a semi-solid medium, and ceramic beads dipped into the pre- and post-challenge monkey sera were spaced equidistantly on the overlay. Quantitation of the serum neutralizing antibody content was accomplished by measurement of the zone of plaque inhibition surrounding the beads. Zones of 10-12 mm are indicative of minimal neutralizing antibody content, whereas zones of 14 mm and larger indicate significant levels of circulating antibody.

Based upon an analysis of the data for each group of monkeys, the following conclusions were drawn:

- a. Subcutaneous inoculation of the Rhesus monkey with Onyong-nyong virus did not produce a demonstrable viremia or signs of an overt illness. CF, HI and NA responses, likewise, were not observed either after initial or homologous challenge.
- b. Challenge by the subcutaneous route produced patent viremias lasting from 2 to 5 days with CHIK-E103, BAH-306, Mayaro-B, and VEE (attenuated), and signs of overt illness accompanied by a temperature rise were consistently observed.
- c. Challenge by the subcutaneous route with these agents afforded solid protection against any sign of overt illness or a demonstrable viremia subsequent to a second challenge with the homologous agent.
- d. Extensive cross-protection patterns were observed to occur among CHIK-E103, BAH-306, and Mayaro B.
- e. A moderate degree of cross-protection was observed to occur among CHIK-E103, BAH-306, Mayaro-B, and VEE (attenuated), as indicated by significant reduction in plasma viremia titers.
- f. Three subcutaneous doses of killed EEE (Eastern equine encephalomyelitis) and WEE (Western equine encephalomyelitis) alone, or in combination with the VEE (attenuated), failed to afford or confer any demonstrable protection against African

or Asian Chikungunya, or Mayaro B viruses.

g. Results of the CF and HI tests showed extensive serological crossing among the agents of Chikungunya, Mayaro B, and Venezuelan encephalomyelitis.

h. Results of the bead neutralization test (BNT) showed the presence of rather broad protection patterns among African, Asian, and Indian* strains of Chikungunya, Mayaro B and Onyong-nyong.

i. Plasma viremia studies in suckling mice and tissue culture systems indicated that peak circulating virus titers occurred between the second and fourth day.

j. Frequently, multiple challenge techniques provoked rather strong anamnestic (heterotypic) reactions.

Two lots of formalin-inactivated vaccines prepared in African Green monkey kidney tissue culture with CHIK-168 virus (African strain) have been assayed in mice. Excellent protection against an IC challenge with the homologous agent was demonstrated in mice vaccinated with the two lots of CHIK-168 vaccine. Bead neutralization tests gave evidence of strong protective interrelationships between CHIK-E103, BAH-306, C-266 (Indian strain), and to a lesser degree, Mayaro B, as shown in the following table:

Bead Neutralization Test (Porterfield Technique)

Serum Tested	CF (168)	HI (168)	Neutralization Test ⁷				
			CHIK-168	CHIK-E103	BAH-306	C-266	Mayaro B
7 da post 1	0	0	10p	0	0	0	0
7 da post 2	8	40	15	12p	11p	9p	0
7 da post 3	8	40	17	13	15	14	10p

⁷ Zone of virus inhibition measured in mm.

p=partial inhibition.

On the strength of these data a sixth group of Rhesus monkeys has been vaccinated with this preparation and will be challenged with CHIK-168, CHIK-E103, BAH-306, C-266, and Mayaro B.

*C-266, a strain of Chikungunya-like virus isolated from a 4½ year old female in Calcutta, India, November 1963. Provided through the courtesy of Dr. V. K. Shah (NIH Fellow).

2. Rift Valley Fever.

During this report period 1400 doses of Rift Valley fever (RVF) virus vaccine were provided to the East African Virus Research Institute, Entebbe, Uganda; Ft. Detrick, Md., and other interested agencies with personnel at risk, with no untoward reactions reported.

Serum neutralization tests are being performed regularly on sera from vaccinated personnel which are forwarded by the above named agencies.

Six sera obtained from chimpanzees at Yerkes Laboratories, Florida, were screened for neutralizing antibodies against RVF virus. All were negative.

Summary and Conclusions:

1. Selected Group A arboviruses within the Chikungunya complex have been investigated for the purpose of determining which agent would be most suitable for the production of a vaccine offering broad spectrum protection within the group. Forty two Rhesus monkeys were utilized for this investigation. Pre- and post-challenge sera from the monkeys were examined by CF, HI, and bead neutralization techniques. Broad protective interrelationships were observed to occur among these agents. The excellent protection patterns observed in preliminary assays of two experimental vaccines prepared with CHIK-168 indicated that a vaccine trial in monkeys would be feasible and this phase is currently being carried out.

ARMY RESEARCH TASK REPORT		REPORTS CONTROL SYMBOL CSCRD-6(R2)
ACCESSION NUMBER 36172	PROJECT, TASK, OR SUBTASK NO. 3A012501B8130503	
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D. C., 20315	2. FUNDING AGENCY Army Medical R&D Command Office of The Surgeon General Washington, D. C., 20315	
3. CONTRACTING AGENCY NA	4. CONTRACTOR AND/OR GOV'T LABORATORY A Walter Reed Army Inst of Rsch Walter Reed Army Medical Center Washington, D. C., 20012 723-1000, Ext. 3552	
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Einheber, A., Ph.D., Dept of Germfree Rsch, Div of Basic Surgical Rsch, WRAIR, WRAMC, Washington, D. C., 20012 576-3483 or Interdepartmental Code 198, Ext 3483 See Continuation Sheet		
6. TITLE OF: PROJECT <input type="checkbox"/> TASK <input type="checkbox"/> SUBTASK <input checked="" type="checkbox"/> Responses of germfree animals (U)		
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964		
8. RESUME' (U) <u>Germfree, defined-flora, and conventional(ized) animals were examined to determine their constitution, physiology, and response to various stressors. The goals of these studies are to (1) define the host-parasite relationship, (2) determine the role and mechanisms of action of bacteria and their products, and (3) modify and regulate indigenous microflora to the advantage of the host. The enlarged cecum of the germfree animal was studied with regard to its mode of filling, innervation, and content. Germfree and conventional animals were compared in: serotonin and histamine content of their tissues; tryptophane pyrrolase activity and its response to d- & l- tryptophane (and in uremia); morphological and humoral response of the lymphatic system to radiation; survival, histopathology, and biochemistry in uremia; response to antibiotics; response to parasitic infestation; and response to injury by limb ischemia, bowel ischemia, burns, and radiation. The process of conventionalization with bacteria was studied. Observations are being made on inflammation, liver regeneration, and spontaneous tumors in germfree animals. Techniques were developed for anesthesia, gastrostomy, and skin grafts adaptable to germfree isolators. Progress is being made toward developing a colony of germfree hairless mice. Technological improvements were made on germfree and clinical isolators.</u>		
9. KEY WORDS Germfree, bacteria, antibiotic, shock, irradiation, pathology		
10. SUPPORTING PROJECTS Not Applicable		
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet	12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet	
DA FORM 1309R 1 June 63 PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of ____		

ACCESSION NO.

36172

ARMY RESEARCH TASK REPORT

13. PROJECT, TASK OR
SUBTASK NUMBER

11	22	23 24	25 26	27 28	29
3 A 0 1 2 5 0 1 B 8 1 3	0 9	6 3			

14. DATE OF REPORT (30-33)

30	33	34
0 6 6 4	4	

15. SECURITY OF WORK (34)

16. TYPE OF REPORT

35	36	47 48 49 50 51	52	55
3			1 2 6 3	

17. SCIENTIFIC FIELD

 a. Topical Classific. (56-61)
 b. Functional Class (62-64)

56	61	62	64
0 1 0 6 0 2			

18. OSD CLASSIFICATION

(65-66)

19. R&D CATEGORY (67)

65 66	67
R R	1

20. CONTRACT NUMBER

11 12	13 14	15	17	18	21	22	26	27
D A								

21. GRANT NUMBER

28 29	30	33	34 35	36	38	39 40	41	45	46
D A							G		

22. ESTIMATED COMPLET.
DATES

47	51	52	56	57	61	62	66	67	71
1	C O N T	2		3		4		5	

23. PRIORITY (11-14)

24. PROGRAM ELEMENT
(15-26)

11	14	15	26
	2	6 • 1 1 • 2 9 • 0 1 • 1	

25. CMR&D CODES

27	29	30	32	33	35
N /					

26. CDOG REFERENCE

a. Paragraph No. (36-44)

b. Functional Group (45)

36	39	40	41 42	43 44	45
1 4 1 2					6

27. FUNDING

a. Est. Total Cost (11-15)

b. % Spent Intern. (16-18)

" " Extern. (19-21)

c. Total Obligation (22-26)

d. Progrmd. Cur. FY (27-33)

e. " " " +1 (34-40)

f. " " " +2 (41-47)

g. " " " +3 (48-54)

h. " " " +4 (55-61)

i. " " " +5 (62-68)

j. " " " +6 (69-75)

k. Total Man Years of
Effort (76-78)

11	15	16	18	19	21	22	26
		1		2			
27 28	29	33	34 35	36	40		
41 42	43	47	48 49	50	54		
55 56	57	61	62 63	64	68		
69 70	71	75	76	78			

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

- (A) Bauer, H., M.D., Dept of Germfree Research, Div of
Basic Surg Rsch, WRAIR, WRAMC, Washington, D. C., 20012
576-3483 or Interdepartmental Code 198, Ext 3483 49
- (A) Brown, N. D., B.S., Dept of Germfree Research
Div of Basic Surg Rsch, WRAIR, WRAMC, Washington, D. C., 20012
576-3483 or Interdepartmental Code 198, Ext 3483 49
- (A) Burns, W., M.D., Dept of Germfree Research, Div of
Basic Surg Rsch, WRAIR, WRAMC, Washington, D. C., 20012
(Grant - Georgetown Univ School of Medicine) 49
- (A) Carter, D., Capt, MC, Dept of Germfree Research, Div of
Basic Surg Rsch, WRAIR, WRAMC, Washington, D. C., 20012
576-3483 or Interdepartmental Code 198, Ext 3483 49
- (A) Hysell, D. K., 1st Lt, VC, Dept of Germfree Research, Div of
Basic Surg Rsch, WRAIR, WRAMC, Washington, D. C., 20012
576-3483 or Interdepartmental Code 198, Ext 3483 49
- (A) LaConte, M. L., Maj, ANC, Dept of Germfree Research, Div of
Basic Surg Rsch, WRAIR, WRAMC, Washington, D. C., 20012
576-3483 or Interdepartmental Code 198, Ext 3483 49
- (A) Rosen, H., Ph.D., Dept of Germfree Research, Div of
Basic Surg Rsch, WRAIR, WRAMC, Washington, D. C., 20012
576-3483 or Interdepartmental Code 198, Ext 3483 49
- (A) Wren, R. E., B. A., Dept of Germfree Research, Div of
Basic Surg Rsch, WRAIR, WRAMC, Washington, D. C., 20012
576-3483 or Interdepartmental Code 198, Ext 3483 49

REPORTS. Annual Progress Report, Walter Reed Army Institute
of Research, 1 July 1963 - 30 June 1964.

Bauer, H., Horowitz, R. E., Watkins, K. C., and Popper, H.: Immuno-
logic Competence and Phagocytosis in Germfree Animals with and with-
out Stress. J.A.M.A., 187:715-718, 1964.

Bauer, H., Horowitz, R. E., Faronetto, F., Einheber, A., Abrams,
G. D., and Popper, H.: Influence of the Microbial Flora upon Res-
ponse of Serum Gamma Globulin and Lymphatic Tissue to Irradiation.
Studies in Germfree Mice. Lab. Invest., 13:381-388, 1964.

REPORTS - continued:

Beaver, D. L., Ashburn, L. L., McDaniel, E. G. and Brown, N. D.: Lipid Deposits in the Lungs of Germfree Animals. Arch. Path., 76: 567-570, 1963.

Burns, W., Bauer, H. and Einheber, A.: Quantitative Morphology of the Thymus in Normal and Incidental Germfree Mice. Fed. Proc., 23: 547, 1964.

Dobernack, R. C., Nunn, D. B., LaConte, M. L., Kimler, A. and Pulaski, E. J.: Foracaval Shunt Operation in a Surgical Isolator: A Study of Cultures during Operation and Wound Healing Thereafter. Milit. Med., 129:259-263, 1964. (See Dept of Nursing Annual Report, Project 3A012501A803, Military Internal Medicine, Task 01, Internal Medicine, Subtask 04, Military Nursing)

Formal, S., Abrams, G. D., Schneider, H. S. and Laundry, R.: Penicillin in Germfree Guinea Pigs. Nature, 198:712, 1963.

Horowitz, R. E., Bauer, H., Paronetto, F., Watkins, K. C., and Popper, H.: The Effect of the Microflora on Lymphatic Tissue Reactions following Whole-Body Irradiation. Studies in Germfree Mice. Am. J. Path., 42:35a, 1963.

Horowitz, R. E., Bauer, H., Paronetto, F., Popper, H., Abrams, G. D. and Watkins, K. C.: The Response of the Lymphatic Tissue to Bacterial Antigen. Studies in Germfree Mice. Am. J. Path., 44: 747-761, 1964.

Levenson, S. M. and Tennant, B.: Germfree Research: Metabolic and Nutritional Studies. Proceedings of the XVI International Congress of Zoology, Aug. 20-27, 1963, 3:148-154, 1963.

Levenson, S. M., Trexler, P. C., LaConte, M. L., and Pulaski, E. J.: Application of the Technology of the Germfree Laboratory to Special Problems of Patient Care. Am. J. Surg., 107:710-722, 1964.

Rosen, H.: Combustion Method for Estimation of Deuterium in Animal Tissues. Anal. Biochem., 7:215-217, 1964.

ANNUAL PROGRESS REPORT

Project No. 3A012501B813 Title: ARMY MEDICAL BASIC RESEARCH IN
LIFE SCIENCES

Task No. 05 Title: Immunology

Subtask No. 03 Title: Responses of germfree animals

Description:

Study phases are in progress or are completed on:

1) The germfree animal, its technology, production, rearing, maintenance, and nutrition; to evaluate and exploit its potential in biologic research.

2) Comparative analyses of the constitution (anatomy and biochemistry) and function (behavior, physiology, metabolism) of germfree, defined-flora, and conventional(ized) animals.

3) Comparative analyses of the responses (constitution and/or function) of germfree, defined-flora, and conventional(ized) animals after challenge with physical, chemical or viable noxae, or combinations thereof; to learn the possible role of the indigenous and environmental microorganisms (and their products), and the effects of their modification and/or control. One of the aspects of this phase of investigation is the use of germfree, defined-flora, and conventional(ized) animals to attempt to assess the Role of Bacteria in Shock, the other subtask of this Department.

Progress:

Animal Production and Utilization:

During the year 1 July 1963 to 30 June 1964, 1,630 animals were used for investigative purposes. The source and species of animals are as follows:

Born and reared germfree or delivered by Caesarean section in this Department:

Rats	130
Mice	46
Guinea Pigs	38

Obtained from Charles River Breeding Laboratories:

Rats	400
Mice	1016

Establishment of a Germfree Colony of Hairless Mice:

Germfree hairless mice could be a valuable biologic research tool. In burn studies, wound healing, general surgical procedures, and others, it would greatly facilitate surgical preparation and post-surgical observations. A source of conventional hairless mice is readily available in the Walter Reed animal colony. On several occasions, we have attempted to caesarian-derive and later suckle a nucleus group of such mice for establishment of a germfree colony but experienced a high incidence of cannibalism from ND-2 foster mothers. By using ICR females as foster mothers, we succeeded in deriving three germfree female hairless mice. We then bred the germfree hairless females to a germfree ICR male and were successful in getting several litters. We have observed several color variations in the offspring, namely, white, black, cinnamon, brown and agouti. By continual breeding, we have acquired 3 more hairless mice and several definite color variations. From the Animal Research Unit at Fort Knox, Kentucky, where this strain of hairless mice originated, we have learned that best results will be obtained if a hairless male is bred to a female with hair which has the recessive gene for hairlessness, because the hairless females have a higher incidence of inverted nipples. We anticipate being able to use this breeding procedure with our mice in the very near future. Barring unforeseen difficulties, we should be producing small numbers of the germfree hairless mice soon. The germfree hairless mice have exhibited none of the rough skin appearance or eye disorders common among their open-room conventional counterparts.

Evaluation of A New Pellet-Form Diet for Germfree Rats:

Encouraging results were obtained on the growth response of open-room conventional rats fed a sterilized new pellet-form L-356 diet. (See Annual Progress Report, 1962-1963). However, satisfactory growth of conventional rats fed these pellets did not necessarily insure that such would obtain with germfree rats. We therefore randomized and placed two groups of weanling Fischer germfree rats on one of two forms of steam-sterilized, commercially prepared L-356 diet ad libitum. Each group contained three females and five males, housed in a stainless steel Reyniers isolator. The males and females were caged separately in groups with adequate cage space available.

In group I, fed the pelleted L-356, the average weight gain over the 47-day period was 57.3 grams, a 1.2 grams average daily gain. Group II, fed only powdered L-356 diet, gained a total average of 104 grams over the 47-day period, an average daily gain of 2.2 grams per day. Initially, the average body weight of the group I rats was 76.9 grams while the

group II rats weighed an average of 72.0 grams. These weights may be contrasted with the final body weights of 134 grams in group I and 176 grams in group II, respectively. Little or no difference was noted in the growth response between the males and females.

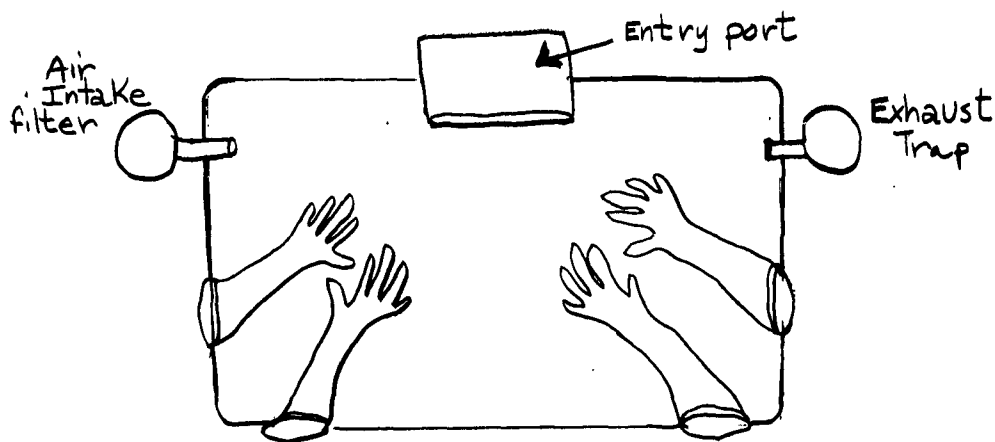
It would appear that steam-sterilized pelleted L-356 diet is inferior to the steam-sterilized powdered L-356 diet in routine use, and further evaluation of the pelleted diet has been suspended.

Germfree Technology:

Need has arisen for new equipment or modification of pre-existing equipment and we have devised and fabricated certain items, with improved functional attributes.

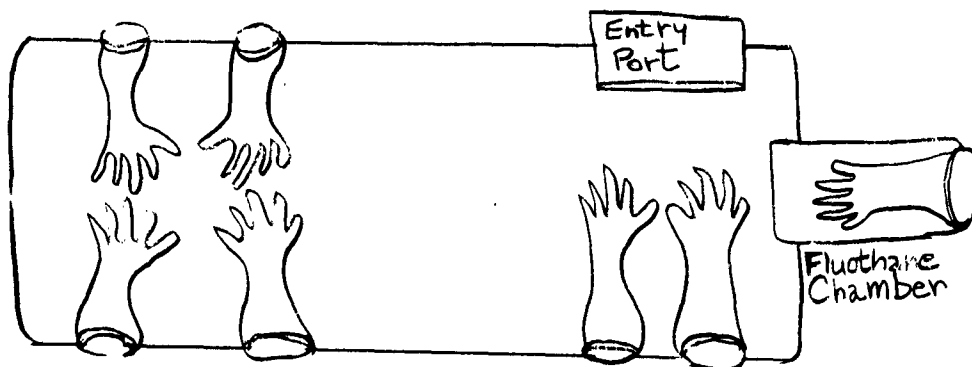
Isolator Modification - Most germfree isolators, both steel and plastic, have only one set of gloves unless they are special examining or surgery tanks. Manipulations of mice usually require at least two sets of gauntlets for adequate restraint and accuracy. Through Mr. Vincent Butler's assistance, we have made several modifications which have greatly improved our utilization of the basic plastic isolator. A modification has been made on the 24"x24"x24" plastic isolator by attaching two sets of rubber gauntlets instead of the original one set. These gauntlets are situated diagonally from each other and the entry port.

The diagram below illustrates this.



By placing the gauntlets about the corners of the isolator, more facile and complete utilization of the isolator space is obtained. The entry port is accessible to either set of gauntlets. We have successfully used this modified tank in several experiments, e.g., tourniquet shock, mouse back burn, I^{131} injections, and radioactive thymidine injections. Its small size and weight contribute to its maneuverability. Reflection of light by the plastic and the resulting glare makes for less than ideal vision needed for meticulous surgery; insertion of a clear plastic viewing port should overcome this and such models are being fabricated.

The other isolator modification is on a 72"x24"x24" plastic isolator. This isolator permits two men to assist one another in surgical procedures, etc., and a third man to handle transferring of animals into the isolator, anesthetizing and preparing of the animals prior to surgery. The diagram below illustrates this.



In this isolator, we have placed a rigid plastic Fluothane chamber with single gauntlet. By adding a clear, rigid plastic viewing port for the surgeon, this isolator will be further improved.

Animal-Ejector-Sleeve - The need for and design of this device were discussed in detail in last year's annual progress report. In this past year, the ejector-sleeve has proven successful in serial removal of animals from the germfree isolators. The present design incorporates the use of flexible plastic film because of ease of construction. We are developing a rigid plastic model that is less susceptible to puncture and more durable. The modification of the ejector sleeve referred to last year to make a dead end tube has been accomplished using the rigid plastic tubing for the dead end. This dead end sleeve has been used on many occasions to facilitate whole body radioisotope counts on mice.

Mouse and Rat Scatter-Proof Self-Feeder - We have been trying to devise a mouse and rat feeder and waterer which would permit accurate measurement of consumption for experiments in which food and water consumption might be an important variable. The design of our pilot model feeder is as follows: A central feed cylinder contains the food which is accessible to the mice only through a series of holes at its base; a larger outer cylinder acts as a reservoir for spilled food. This outer cylinder has holes corresponding to those of the food cylinder so that the mice can eat by sticking their heads into the eating ports. By weighing the feeder before and after the mice have eaten, an accurate measurement of food consumption can be obtained. Mice learn to use the feeder very quickly. However, gravity is not sufficient to maintain a steady flow of food. We have been using finely ground L-356 diet to prevent the mice from carrying away any large chunks of food in their mouths. An additional modification under test is to add an agitator rod in the center of the food column, propelled by a spring or electric motor to assure continuous flow of food to the eating ports.

A watering device built on the same principle of a central water column surrounded by an enclosed reservoir to catch spillage is simpler, as gravity will be sufficient to provide flow to the drinking ports.

Development of Clinical Isolators

See Project Report 3A012501A803 Military Internal Medicine, Task 01 Internal Medicine, Subtask 04 Military Nursing.

Anesthesia:

Anesthesia poses a problem in germfree systems. The flammable characteristics of ether preclude its safe use. Chloroform's high toxicity and the unavailability of a metering device limit its use. It is difficult to predetermine precise dosage of an injectable agent like Nembutal (since the germfree animal's enlarged cecum and its contents comprise a large but varying percentage of the body weight). Presently, we are investigating Fluothane. Its toxicity appears to be between ether and chloroform, it is non-flammable, and there is a commercial metering device (Mark II Fluotec) which makes depth of anesthesia more precisely controllable. The potential liver-damaging effects of Fluothane are undetermined. Major Everett Mosley, an anesthesiologist assigned to WRAIR, assisted us in the evaluation of Fluothane. Mice were exposed to varying concentrations of Fluothane once or twice weekly. After 1 to 4

exposures, the animals were then sacrificed and the viscera subjected to pathologic examination. There was noted a mild, transient-type damage in some of the livers but this damage could not be correlated with number of exposures to anesthesia or concentration of anesthesia. It may reflect individual variation among mice. Fluothane, thus far, appears to be safe for our purposes.

In the course of these studies, we developed an anesthesia chamber which is attached to a plastic isolator. This chamber is made of acrylic plastic which can be chemically sterilized by peracetic acid. It has its own fiberglass inlet filter, germicidal exhaust trap and one gauntlet. It is essentially sequestered from the rest of the large isolator to which it is attached and virtually confines the Fluothane within its boundaries. This chamber is adequate for light anesthesia and for induction purposes. Our standard air-filtration conduits are adequate for the removal of bacterial contaminants from the air-Fluothane mixture.

A method for using Fluothane anesthesia in a steel surgical isolator has also been devised. Air is mixed with Fluothane in a Mark II Fluotec to provide the desired concentration. This mixture is then fed through a fiberglass filter into the steel isolator. Within the isolator, it can be fed into a small induction chamber which is exhausted separately. After surgical anesthesia has been induced, the mixture can be channeled to a nose cone which fits both rats and mice for maintaining light surgical anesthesia. This system has been successfully used on germ-free and conventional rats.

To summarize, an effective system for using Fluothane, a non-flammable, carefully controllable, gas anesthetic has been devised and used. This has great value for surgical and other procedures carried out on germfree animals.

Gastric Physiology: Gastrostomy.

A method was sought for providing a permanent gastric fistula in germ-free and conventional rats through which various agents or liquid diet might be administered, and from which samples of the gastric contents might be removed for studies of gastrointestinal physiology.

The method of Komarov, *et. al.* (Proc. Soc. Exp. Biol. Med., 112:451, 1963) was modified. Metal gastric cannulas, made for us by the Division of Instrumentation, WRAIR, have been implanted and have functioned successfully for up to 5 months.

Amino Acid Metabolism:

All amino acids of living animals are left-handed, but there is no firm clue as to why this should be so. This fact, is however, so ingrained in the consciousness of the world of biochemistry that the "left-handed" or "l" amino acids are universally known as the natural isomers. It was discovered about 30 years ago, however, that laboratory animals such as the rat can survive in good health when fed some essential amino acids (such as tryptophane) in the "d" or unnatural form along with the other amino acids in the "l" form. (Berg, C.P., *Physiol. Rev.* 33:143, 1953). Painstaking analyses have failed to disclose even a trace of d amino acids in the body proteins of the animals to which they were fed. They must have been converted to the l form before utilized. The mechanism of this conversion is not yet known. Interest at first centered on a search for enzymes which could use d amino acids as substrates; the only one of consequence thus far discovered is d-amino acid oxidase, that can oxidatively deaminate all the d amino acids. The keto derivatives might therefore be available for reamination to yield the l forms. There is skepticism, however, that this is the major mechanism for d amino acid inversion for the following reasons: (1) this enzyme is found almost exclusively in the kidneys, and since the liver is the major locale for amino acid metabolism, the renal location of the d-amino oxidase would seem inappropriate; (2) there are wide variations in amounts of d-amino oxidase among animal species without concomitant variations in their abilities to utilize the d amino acids; (3) d-amino acid oxidase is more active toward some amino acids than toward others; this variation of activity, however, does not correlate with the ability of the animal to metabolize one or the other of the d amino acids; (4) there is doubt whether d amino acids are actually the natural substrate of d amino oxidase at all; it has been suggested that the natural substrate of this enzyme has never been discovered, and that its activity toward the d amino acids is an in vitro artifact. (Meister et al, *J. Nat. Cancer Inst.* 24:31, 1960).

In contrast to mammals, many bacterial species have enzymes which can deaminate, transaminate and decarboxylate d-amino acids, (Thorne, C.B. and Molnar, D.M., *J. Bact.* 70:, 1955). It occurred to us that the d amino acids might be metabolized mainly through the intervention of fecal bacteria rather than by tissue enzymes. Accordingly, we have laid out a plan, by the use of conventional and germfree rats, to determine what part of the metabolic fate of metabolizable d amino acids is due to bacteria, and what part, if any, to animal tissues.

✓ For our initial studies, we chose tryptophane for the following reasons: 1) d-tryptophane is utilizable by the rat, and probably by humans; 2) tryptophane metabolism makes itself felt in an area in which this laboratory has background data, the "biogenic amines", e.g., histamine and serotonin; 3) tryptophane is involved in one of the very few possible enzyme inductions by substrate in mammals; 4) Tryptophane is an essential amino acid both for the rat and human.

The plan embodied a four-fold approach consisting of: 1) measurement of rates of absorption of d and l-tryptophanes from the gut; 2) possible induction of the enzyme tryptophane pyrrolase as a measure of blood l-tryptophane; 3) excretion of the metabolites kynurenic and xanthurenic acids after feeding d and l-tryptophanes; and, 4) excretion of C^{14} into expired air and urine, as well as incorporation into proteins, after giving tracer doses of C^{14} tryptophane in the d and l forms.

The first two experiments have been completed. Table 1 shows the percentile results of the absorption studies. In both conventional and germfree rats, there was about 30% more of the d isomer remaining in the G.I. tract after 3 hours than of the l isomer. It appears that the l isomer is similarly absorbed by both conventional and germfree rats, but the rate of absorption is over 80% slower in the germfree. With respect to the d isomer, the absorption rate difference is also about 80%, but there are also differences within the segments of alimentary tract, notably stomach and small intestine. An incidental finding was that the germfree cecum normally has large amounts of tryptophane, amounting to about 3.5 mg.

This experiment does not conclusively indict bacteria as mediators in d-tryptophane metabolism, but is consistent with this possibility. It also shows that a) the d isomer need not be inverted prior to absorption and b) the absorption capability of the germfree toward either tryptophane is inferior to that of the conventional animal. These animals received unphysiologically large amounts of tryptophane (100 mg), and one cannot assume that physiological amounts of either tryptophane are handled with the same dispatch by the germfree as by the conventional rat. Tracer studies with C^{14} tryptophane should be decisive.

Table 1

Recovery of Tryptophane After 3 Hours in Rats Fed 100 mgm
Tryptophane in 5 ml. Saline

	<u>Germfree</u>	<u>Conventional</u>
<u>L-Tryptophane</u>		
Total Recovered	12.8%	6.8%
% in: Stomach	82.9%	82.4%
Small Bowel	5.3%	6.4%
Cecum	11.8%	11.2%
<u>D-Tryptophane</u>		
Total Recovered	17.6%	10.0%
% in: Stomach	50.9%	21.7%
Small Bowel	8.7%	47.4%
Cecum	40.4%	31.2%

The induced enzyme study was undertaken in the expectation that substrate induction would be accomplished by l-tryptophane only, and thus yield information regarding the amount of the fed d-tryptophane which was converted to the l form. It is known that a number of compounds can induce the enzyme hormonally, but Knox (Brit. J. Path. 32:462, 1951) on the basis of experiments with d and dl-tryptophane, concluded that the d isomer was not one of these compounds. Reference to Table 2 shows that Knox was unfortunately almost certainly incorrect in his conclusions regarding the inefficacy of d-tryptophane in the hormonal stimulation of the enzyme. Both groups of rats responded to the d-tryptophane with elevations of tryptophane pyrrolase about one-half as great as those observed after the l-tryptophane. The actual elevations after d-tryptophane are probably even greater, since the absorption of the l-tryptophane was faster, thus leading to a higher blood level. It seems highly unlikely that the d-tryptophane-induced elevations could have resulted from massive inversion in so short a time, and probably denotes hormonal response to d-tryptophane. Reference to Table 2 shows that

the enzyme did achieve higher levels after d-tryptophane in the conventional as compared with the germfree rat, but this most likely reflects differences in absorption than inversion. Thus, while the enzyme study yielded valuable information regarding the mechanism of enzyme induction, it is not a tool for the evaluation of d-tryptophane metabolism.

Table 2

Tryptophane Pyrrolase of Livers 3 Hours After Feeding 100 mg
Tryptophane in 5 ml. Saline

Units: μ m Kynurenine Formed /hr/gm dry Liver

<u>l-Tryptophane</u>			
<u>Animal No.</u>	<u>Germfree</u>	<u>Animal No.</u>	<u>Conventional</u>
2	52.1	11	55.1
3	51.1	12	41.3
4	43.5	13	55.1
		14	20.6
Mean	48.9		48.0
<u>d-Tryptophane</u>			
5	32.0	15	26.4
6	26.7	16	33.7
7	19.1	17	24.1
8	11.2	18	29.7
Mean	22.3		28.5
<u>Saline Controls</u>			
9	Negligible	19	Negligible
10	" "	20	" "

The study involving tryptophane metabolites is in its beginning stages, as are the C¹⁴ tracer studies.

This study should have practical consequences in the field of nutrition since the availability of pure synthetic amino acids has made practical the preparation of non-antigenic diets for human consumption. The choice of l or dl-amino acids in any specific case might then be made on the basis of the bacterial flora found in the patient's G.I. tract. In addition, it is known that in vitro bacterial l-amino acid decarboxylases can be induced by large amounts of the amino acids themselves. It is possible and even probable that this can also occur in vivo. We hope to be able to find whether the bacterial d-amino acid enzymes are also adaptive in vitro and in vivo. Since the feeding of large amounts of an l-amino acid would presumably result in its eventual wasting by the mechanism of bacterial decarboxylation, it might be possible then to switch to the corresponding d amino acid to which the bacteria have not adapted. In this way, by the alternate feeding of d and l-amino acids, maximal nutrition might be achieved.

The Enzymes: Tryptophane Pyrrolase.

Livers of rats sacrificed after 48 or 72 hours of uremia were analyzed for tryptophane pyrrolase activity. This enzyme is induced in mammalian livers by its specific substrate l-tryptophane. Its activity is also increased in response to stress situations, and this response is apparently mediated by adrenocortical steroids.

The following values were obtained for the activity of the enzyme in μ M of Kynurenine per hour per gram dry weight of liver:

Time, Post-Op., Hrs.	Status	Germfree	Conventional
72	Nephrectomy	15.4	16.6
72	Sham	11.8	10.5
48	Nephrectomy	13.4	18.9
48	Sham	10.5	9.7

When the nephrectomized animals were compared with the shams, regardless of microbial status, the following results were obtained:

Time, Post-Op., Hrs.	Status	Tryptophane Pyrrolase Activity*
72	Nephrectomy	16.1
48	Nephrectomy	15.4
72	Sham	11.3
48	Sham	10.0

* μ M of Kynurenine per hour per Gm. Dry Weight of Liver.

To summarize, uremic rats, regardless of microbial status, have statistically significantly higher tryptophane pyrrolase activity per gm of dry liver weight than sham-nephrectomized rats. The stimulus for higher specific activity of tryptophane pyrrolase is not known. Whether it is a rise in l-tryptophane concentration is not clear. Administration of tryptophane, of all the amino acids, reduces blood glucose concentration by an unknown mechanism. However, we found no significant changes in blood glucose levels among these groups of animals. The rise in tryptophane pyrrolase activity in the uremic rats therefore probably relates to an increased level of adrenocorticosteroids in these animals. Further clarification using nephrectomized, adrenalectomized rats is under way.

Serotonin and Histamine:

Last year, (Ann. Prog. Rept. 62-63), we reported that baseline observations of serotonin and histamine levels in a variety of tissues were being analyzed in germfree and conventionalized rats. During this past year, a large number of fluorometric determinations were completed. Statistical correlations and calculations are still in progress. Comparing germfree and conventionalized rats, the calculated mean tissue levels of serotonin and histamine, on a fresh tissue weight basis were determined to be as follows:

Serotonin $\mu\text{g/g}$ Wet Tissue

Tissue	Germfree			Conventionalized			P*
	No. Rats	Mean	S.E. of Mean	No. Rats	Mean	S.E. of Mean	
Glandular Stomach	20	3.87	0.41	17	3.39	0.47	N.S.
Prox. Small Intestine	20	3.88	0.29	18	3.18	0.29	5%
Distal Small Intestine	19	4.32	0.45	18	3.69	0.41	N.S.
Cecum	20	6.82	0.62	16	6.52	0.54	N.S.
Prox. Colon	19	4.76	1.10	17	3.14	0.79	N.S.
Liver	20	0.35	0.06	18	0.28	0.04	N.S.
Lungs	14	0.79	0.38	13	0.63	0.33	N.S.
Spleen	19	2.28	0.25	18	1.57	0.21	1%
Brain	20	0.31	0.01	18	0.34	0.01	5%

Histamine $\mu\text{g/g}$ Wet Tissue

Glandular Stomach	20	27.47	1.72	17	29.36	1.70	N.S.
Prox. Small Intestine	18	5.27	0.33	16	5.09	0.37	N.S.
Distal Small Intestine	17	6.80	0.46	17	6.16	0.39	N.S.
Cecum	19	13.08	1.13	16	8.49	0.81	0.1%
Prox. Colon	16	8.70	0.84	15	4.42	0.31	<0.1%
Liver	20	1.39	0.18	18	1.52	0.26	N.S.
Lungs	19	5.73	0.49	17	3.54	0.35	5%
Spleen	20	2.70	0.30	17	2.71	0.28	N.S.
Brain	20	0.39	0.07	18	0.46	0.09	N.S.

* t-test; N.S.= Not statistically significant.

The data suggest that serotonin tends to be present in generally higher amounts in the wall of the gastrointestinal tract of the germ-free rat; however, the only statistically significant difference was that for the proximal segment of small intestine. This correlates with the reported finding of a greater serotonin content in the small intestine wall of the germfree animal by Beaver and Wostman (Brit. J. Pharmacol. 19:385, 1962).

Serotonin content of liver, lungs, and spleen of the germfree rat also was generally higher, but to a statistically significant degree only in spleen. By contrast, the serotonin content of brain was greater in the presence than in the absence of a microbial flora.

While in the observations of Beaver and Wostman (Brit. J. Pharmacol. 19:385, 1962) differences in histamine levels of the small intestine and cecum between germfree and conventional rats were equivocal, there was a tendency toward higher values in the conventional animal. Our observations, on the contrary, suggest that the germfree gastrointestinal tract tissue tends to have more histamine than that of its conventionalized counterpart; histamine levels of the cecum and colon were significantly higher in our germfree animals. The significance of the higher histamine levels in the germfree cecum is presently uncertain. The only difference in histamine level obtained in the other tissues examined was that of the lung, germfree animals showing a much higher level.

Investigations of C¹⁴ tryptophane metabolism and other amino acids that are in progress (see elsewhere in report) should contribute to our further understanding of the differences in tissue amine levels that we observe.

Carbohydrate Metabolism: Diabetes.

The studies begun in 1962-63 were temporarily suspended this past year in order to enable completion of the serotonin and histamine analyses. We anticipate resumption of this project.

Inflammation: "Granuloma Pouch" Technique.

Preliminary observations in the use of this technique were presented in Ann. Prog. Rept. 62-63, Development and Maintenance of Germfree Animal Colonies. Further testing has led to discouragement of continuation of these studies at the present time. It became apparent that air-injection into the subcutaneous space of the dorsum of Fischer rats resulted more consistently in uniformly sized and shaped air-pockets

in female than in male rats. This necessitates exclusive use of females and, since we house male and female rats separately in each isolator, creates an unwieldy problem under present circumstances of animal supply. Moreover, air-injection prior to injection of the irritant does not always produce ideal air-pockets, even in females. This creates uneconomical exclusion of such animals from an experimental series. With future expansion of rat supply and increased holding facilities, we will reinstitute these studies.

The Lymphatic System: Morphologic and Humoral Response to Radiation.

Quantitative, histologic, immunocytochemical, autoradiographic, and electrophoretic studies were performed on normal and irradiated germ-free and conventionalized mice in collaboration with the Mount Sinai Hospital, New York. One-third of conventional but no germfree mice were killed by 550 r whole body irradiation. Before irradiation, germfree mice had fewer immunologically active cells in lymphatic tissue and a lower serum gamma globulin than conventional animals. After irradiation, a pronounced immune response developed in the lymph nodes and spleens of all survivors. This was associated with a rise in serum gamma globulin in the germfree but a drop in the conventional mice. Gamma globulin decreases after low-lethal doses of radiation. This has been attributed to protein leakage from the radiation-damaged intestine. Possibly in germfree mice no such loss occurs.

The thymus of germfree mice is smaller and produces fewer cells than its conventional counterparts. Thymus morphology and cell turnover thus correlate directly with immunologic activity which is low in germfree and high in conventional mice. After irradiation, the thymus and lymphatic tissues of germfree and conventional mice recover equally well. Reactions of the thymus and lymphatic tissue to irradiation are therefore modified but not basically altered by the microflora. (vide The Mount Sinai Hospital Annual Rept., 1 May 63 - 29 Feb 64, Grant No. DA-MD-49-193-G24).

Tissue Transplantation: Skin Grafting.

Efforts have been made to adapt a technique described by Gross, Padnos, and Gottfried to use in germfree isolators. (Plast. & Reconst. Surg. 25:421, 1960). Ability to obtain a consistently high percentage of takes has thus far evaded us. A visit to Dr. Padnos at the Waldemar Medical Research Foundation has been arranged. It is hoped that a discussion with him will show us the reasons why we have been unable to reproduce his technique.

Parasitic Infections: Schistosomiasis.

In collaboration with the Department of Medical Zoology (Dr. Elvio Sadun and Mr. John Bruce), studies have been initiated on Schistosoma mansoni infections in germfree mice. These studies were initiated to determine the factors responsible for localization of the adult worms in the portal mesenteric system. Initial trials tested the infectability of germfree mice with sterile S. mansoni cercariae, and the location of adults in the portal circulation.

The results of the first experiment (Table 1) were inconclusive both because very few adult worms developed in the mice and because contamination occurred. It was evident that contamination was due to the fact some cercariae coexisted with bacteria (Staphylococcus albus, Escherichia coli and Aerobacter aerogenes) at the time of penetration into the skin. Contamination occurred despite the fact that cercariae were obtained by sterile technique from snails which had been swabbed with 70% alcohol, placed in a penicillin bath for 24 hours, and coated with sterile paraffin through which a hole was made to permit emergence of cercariae without fecal soiling. The procedure apparently rendered the cercariae non-infective. Techniques of producing sterile cercariae are under study.

Table 1

<u>Group</u>	<u>No. of Mice</u>	<u>No. of Cer.</u>	<u>Days After Exposure</u>	<u>Worms Recovered</u>	<u>Total</u>	
				<u>Male</u>	<u>Female</u>	
Germfree*	10	200	40	3	0	3.0
Conventional	10	200	40	10	0	10.3

*Contaminated with S. albus, E. coli and A. aerogenes at infection with cercariae.

Standardization of technique is also in progress for investigation of the natural history of Plasmodium berghei infection in germfree rats. (See Annual Progress Report, Project 3A012501A818, Task 01, Subtask 20, Parasitic Diseases).

Antibiotics: Antibacterial vs. Non-antibacterial Activities of Chlortetracycline (Aureomycin)

The pharmacodynamics of antibiotics are ascribable to a composite of antibacterial and non-antibacterial activities of the particular agent.

Separation, experimentally, of these activities is difficult, if not impossible, in open-room conventional animals. We have embarked on multidisciplinary studies of the actions of the tetracyclines, beginning with chlortetracycline in germfree, defined-flora, and conventionalized animals. Preliminary observations on the fatty liver-inducing and other effects of the tetracyclines in open-room conventional mice were presented last year.

In a pilot study, germfree mice were divided into an aureomycin treated group and a saline treated group. All conventionalized mice received aureomycin. Dosage of aureomycin was 100 mgm/kg, of the original body weight of the mouse. A comparable volume (10ml/kg) of sterile saline was given to the saline injection group. Daily intraperitoneal injection was repeated for six days. Food (L-356) and water were available at all times. The animals were weighed the day before injections started and on the 2nd, 4th, and 6th injections. Two days following the last injection, the animals were sacrificed and tissue specimens taken.

A high incidence of diarrhea was noted. Several germfree animals died due to torsion of the cecum. This may have resulted from hyperperistalsis brought on by the intraperitoneally injected aureomycin.

Histopathological evaluation indicated the following: There was a mild peritoneal reaction in the aureomycin-injected animals. Aureomycin treatment resulted in an approximately equal degree of fatty metamorphosis in germfree and conventional mice. There was no evidence of impending chronic liver damage and it appeared that the liver could recover on discontinuing the drug. Whether more prolonged aureomycin treatment would result in liver necrosis and fibrosis cannot be extrapolated from these findings. Except for mild peritonitis, the only other finding attributable to aureomycin was mild but definite inflammation and edema of most germfree and conventional ceca. This may be related to peritoneal irritation by the drug. Inflammation was relatively more marked in the germfree cecum and is perhaps related to its greater size, hence, possibly greater surface exposure to aureomycin. Certain of the tissues were unsuccessfully examined for tetracycline fluorescence. There appeared to be only minor fluctuations in body weight. These mice were full grown and possibly we will see more marked differences on repeating the experiment in growing mice. It was discovered at the termination of the experiment that the germfree mice were actually accidentally mono-contaminated with a yeast-like organism of unknown origin. Interestingly, the morphologic appearance of the gastrointestinal tract, in the presence of the yeast, was unmodified from that seen in germfree mice.

Another experiment utilizing younger mice is in progress. Serial sacrifice of animals will be done so that the evolution of liver fat deposition (if it occurs), may be more clearly defined. In addition to pathology, biochemical changes in liver fat, both quantitative and qualitative, and changes in intestinal serotonin and histamine will be evaluated.

Conventionalization: Gastrointestinal Tract.

There are several anatomical differences between the gastrointestinal tracts of germfree and conventional animals, notably the great enlargement of the germfree cecum. The wall of the gastrointestinal tract is also much thinner in the germfree animal, and no inflammatory cells are present in the intestinal lamina propria. The process by which the germfree gastrointestinal tract is converted to the conventional structure by microorganisms, and the microorganisms which are responsible for the change have not been fully revealed.

An experiment to try to elucidate the process of conventionalization was undertaken, using twenty-eight germfree Fischer rats. Esophagostomies were performed on 13 rats in the following manner. The rats were anesthetized with Nembutal i.m. A midline incision was made in the neck and the cervical esophagus was mobilized, exteriorized, and divided. The distal end was oversewn and allowed to retract into the mediastinum. The proximal end was sutured to the skin as an esophagostomy. The anatomic layers were then closed by suture.

Esophagostomized and non-esophagostomized rats were divided into two groups in two isolators. Using aseptic technique, 10% of the body weight in sterile isotonic saline was given subcutaneously every other day to the esophagostomized rats.

One group of animals was contaminated by placing 24 hour broth cultures of E. coli, Proteus, and Streptococcus fecalis in food, water, and bedding. The second group was contaminated with Staphylococcus albus. The animals were removed from the isolators at death or at 7 and 14 days. Cultures were taken from the throat, thoracic esophagus, stomach, upper small bowel, lower small bowel, cecum, colon, rectum, lungs, and mesenteric lymph node of each animal. The tissues were preserved in 10% buffered formalin for subsequent histological examination.

The following results were obtained:

a. Two of 8 tricontaminated esophagostomized animals survived one week. The others died earlier - the cause could not be determined at autopsy - - it is unlikely that they died of starvation. All esophagostomies were patent. The cultures of the two surviving animals showed all three organisms present on all segments taken. Bacterial stains throughout the alimentary tract also revealed microorganisms in the lumen. No change in cecal size was evident.

b. Four of 5 monocontaminated esophagostomized animals survived one week. (The other died earlier - cause of death could not be determined). Staphylococci were cultured from all segments taken. Bacterial stain revealed microorganisms present only in two of the four cecal lumens. No change in cecal size was evident.

c. Eight tricontaminated intact (non-esophagostomized) rats were included. Four were sacrificed at one week and four at two weeks after contamination. All segments cultured were positive for all three organisms. No gross changes were noted.

d. Five monocontaminated, intact (non-esophagostomized) rats were studied. Two were sacrificed at one week and three at 2 weeks after contamination. All segments cultured Staphylococci. No gross changes were noted.

Histological study of the specimens revealed the following:

a. Esophagostomized, tricontaminated rats showed only rare mononuclear cells in the lamina propria of the small bowel, a moderate number of mononuclear cells in the lamina propria of the colon, and a few eosinophils in the cecal mucosa. The histological picture of the bowel of these animals was very similar to the characteristic germfree configuration.

b. Esophagostomized, monocontaminated rats showed a characteristic germfree gastrointestinal tract.

c. Non-esophagostomized, tricontaminated rats showed moderate mononuclear infiltration of the mucosa of the small and large bowel and cecum. Eosinophilia was more marked at 7 days than at 14 days.

d. Non-esophagostomized, monocontaminated animals showed moderate mononuclear and eosinophile infiltration of the small bowel only. This was more marked at 7 than at 14 days.

In summary, microorganisms reached the intestinal lumen of germ-free animals exposed to either the combination of E. coli, Proteus, and Streptococcus fecalis or Staphylococcus albus even when these microorganisms were barred by prior esophagostomy. The magnitude of morphologic response of the small bowel, large bowel, and cecum to the contaminating bacteria declined in that order. The magnitude of the morphologic response of the germfree gastrointestinal tract to the bacteria tested over a period of 14 days was not great. The gastrointestinal tract more closely resembled the germfree state, rather than that which is seen when a greater variety of aerobic and anaerobic organisms are used to contaminate the animal.

The question of the route(s) by which bacteria enter the gastrointestinal tract has been raised. This question applies equally to the conventionalization of germfree rodents and to the manner in which the intestinal microflora become established in the newborn infant. Both are thought to have an underdeveloped immunological mechanism as well as protective mechanisms in the saliva and the gastric secretions which make it difficult for bacteria to pass directly from mouth to colon, but in both instances, bacteria rapidly become established in the large bowel. Further studies are in progress to try to answer the question raised.

The Cecum in Germfree Animals:

The greatly enlarged cecum accounts for as much as 10% and 30% of the total body weight of rats and guinea pigs, respectively. The physiology of the germfree cecum was studied during the present year.

Cecal ablation in newborn guinea pigs - Day-old guinea pigs were anesthetized with 25 mgm/kgm Nembutal (15 mgm/cc) i.m. and a laparotomy was performed observing aseptic precautions. Sham operations consisted of laparotomy, dissection of the cecum, and exposure of the abdominal contents for the period of time required to remove the cecum from the experimental animals. The experimental animals were treated the same way, except that the cecum was resected and closed with a continuous inverting catgut suture. The abdomens were then closed.

Four sham cecectomies were performed. All four animals survived and thrived. At sacrifice 14 days later, no abnormalities were found.

Fifteen cecectomies were performed. All animals died between 4 and 28 hours postoperatively. Autopsy revealed no leakage, peritonitis or other cause of death.

In summary, newborn guinea pigs tolerate anesthesia and surgery, but not cecectomy.

Response of the germfree guinea pig cecum to emptying. Four 1-year old germfree guinea pigs were operated upon in a surgical isolator under Nembutal anesthesia (25 mgm/kgm i.m.). Laparotomy was performed and the cecum was delivered. A 6-0 silk purse string suture was placed in the cecum. The cecum was then incised and its contents emptied into a container. The purse-string suture was then tied and two Lembert sutures placed over it. The laparotomy was then closed in two layers.

Within 24 hours after the ceca had been emptied, the ceca were observed to have quantitatively refilled, whether the animals were allowed to eat and drink or were fasted. Three of the 4 animals died within 24 hours. No leakage or other abdominal pathology was noted.

To summarize, the ceca of germfree guinea pigs refill quantitatively within 24 hours, regardless of whether food or water are taken. Loss of 25% of the body weight into the intestinal tract may be lethal. Studies are in progress to determine the character and source of the intestinal contents.

Innervation of the germfree cecum - In association with the Department of Experimental Pathology, WRAIR, the ceca (of paired germ-free and conventional rats) were removed, processed and stained by routine and histochemical techniques, as well as methylene blue stain.

Auerbach's primary plexus appeared to have grown in proportion to the cecal enlargement. The meshwork, particularly on the anti-mesenteric side, was larger and the ganglia were spaced farther apart. The cell size of the neurons of the germfree rats varied greatly. A decrease in DPNH-diaphorase and acetylcholinesterase activity was detected in Auerbach's ganglia of the germfree ceca. (See Annual Progress Report, Project 3A012501A818, Task 01, Subtask 26, Histopathologic manifestations.)

Comparison of biochemical cecal-contents and blood of germfree and conventional rats - In the experiment on uremia in rats some data are reported on a comparison of serum and cecal electrolyte composition of germfree uremic starved rats. More data are added here to include conventionalized rats.

Blood and Cecal-Contents Urea Nitrogen, Sodium and Potassium in
Starving Germfree and Conventional Rats

Time Post-Op.	Status* & No. Rats	BUN	Total Cecal UN	Serum Na	Total Cecal Na	Serum K	Total Cecal K
Hrs.		mgm%	mgm	mEq/l	mEq	mEq/l	mEq
72	9-CN	658	0.7	149	0.02	9.0	0.013
72	7-GFN	399	30.0	160	0.44	4.9	0.312
	GF/C	0.60	42	1.07	22	0.54	24.0
48	4-CN	313	1.4	160	0.03	4.6	0.028
48	7-GFN	285	21.9	166	0.47	4.9	0.051
	GF/C	0.91	15.6	1.03	15.6	1.06	5.4
72	2-CS	-	1.3	155	0.08	7.6	0.020
72	4-GFS	39	8.2	154	0.62	5.0	0.134
	GF/C	-	6.3	1.0	7.8	0.65	6.7
48	4-CS	27	1.5	142	0.05	4.4	0.015
48	3-GFS	33	16.1	164	1.20	5.5	0.240
	GF/C	1.2	10.7	1.15	24	1.25	16.0

* GF= Germfree; C= Conventionalized; N= Nephrectomized; S= Sham-nephrectomized.

In summary, the germfree cecum seems to be atonic relative to the conventional cecum. The cause is not clear, but it can be overcome by multicontamination with the cecal contents of open-room conventional animals.

The germfree cecum apparently fills rapidly from above when emptied, even if the animal is denied food and water. Accumulation of this volume of gastrointestinal secretions in a short period of time may be lethal.

Certain biochemical constituents of the cecal contents of germfree animals vary considerably from that of conventional animals. The cecal contents of germfree animals also vary considerably from the serum in electrolyte composition. This suggests that the thin wall of the germfree cecum does not behave as a simple semi-permeable membrane.

Further studies are in progress.

Uremia in Germfree and Conventional Rats

Renal failure continues to be a significant clinical problem. Its pathogenesis is not completely understood. Salisbury and others have reviewed a number of hypotheses concerning the uremic syndrome. A number of these include the effect of bacteria or bacterial factors.

We therefore set out to study the response of germfree animals to uremia as a basic problem which might find clinical application in the treatment of uremia and as a model in which the substances normally excreted by the kidney of the germfree animal might be studied.

All animals used were germfree and conventionalized Fischer strain rats obtained from the Charles River Breeding Laboratories and handled according to standard departmental methods. Twenty-four hours prior to surgery, food was withheld and the rats given 10% dextrose in 0.9% NaCl to drink. For surgery, the animals were transferred into a steel surgical isolator. They were anesthetized with 40 mgm/kgm Nembutal (15 mgm/cc) i.m. Bilateral nephrectomy was performed via a midline laparotomy. Care was taken not to injure the adrenals. The wound was closed in two layers and the animals were observed in individual cages. No food or water were allowed postoperatively.

Two non-current survival studies were carried out with the following results:

1

Status	Sex	No. Rats	Mean Survival Time - Hrs.	SE Mean, Hrs.
Germfree	M	4	122.0	3.7
Conventional	M	4	89.0	5.2

2

Germfree	M	7	131.5	5.8
Germfree	F	4	131.0	8.0
Conventional	M	6	68.3	6.2
Conventional	F	3	67.6	14.3

Sham nephrectomies were done with the second study. The animals were treated in exactly the same manner, but the kidneys were left in place. They were observed until they died of starvation.

Status	Sex	No. Rats	Mean Survival Time, Hrs.	SE Mean, Hrs.
Germfree	M	6	355	9.8
Germfree	F	4	251	11.1
Conventional	M	7	526	25.6
Conventional	F	4	413	41.8

Uremic germfree rats consistently outlived their conventional counterparts. There was no overlap. The differences observed were statistically significant.

The situation was reversed with the shams. The conventional rats outlived their germfree counterparts. This difference was statistically significant. The males outlived the females in each group of shams - these differences were also significant.

The rate and amount of weight loss were the same in germfree and conventional animals.

Autopsies were performed on all animals. No lesions were noted grossly. The shams showed marked emaciation. The germfree rats at death still had large ceca.

All tissues were preserved in 10% buffered formalin. No lesions were noted in the shams. The myocardium and aorta of the germfree uremic rats showed extensive calcification. This was not seen in the conventionals. In the ceca of the conventional uremic rats of group 2, necrotizing arteritis and mucosal ulceration with surrounding inflammation was observed. This lesion was similar to that seen in uremic colitis in humans. It was not seen in the rats of group 1, which did not have as part of their intestinal flora the Staphylococcus albus which was present in group 2. None of the germfree rats were found to have this lesion present.

A third group of uremic germfree and conventional animals, prepared as described above, were studied. Half were sacrificed at 48 hours and half at 72 hours. The animals were sacrificed by anesthetizing them with ether, performing a laparotomy and cannulating the abdominal aorta.

They were exsanguinated and the blood was collected and used for chemical determinations. The cecal contents were removed and frozen for chemical determinations. The tissues were preserved in 10% buffered formalin. The following chemical determinations have been made:

Mean Blood Values

<u>Time Post-Op., Hrs.</u>	<u>Status*</u>	<u>Glucose, mgm%</u>	<u>Protein, gm%</u>	<u>Hemolysis</u>
72	CN	102	5.8	3+
48	CN	89	4.6	0-1+
72	CS	105	6.4	2+
48	CS	101	5.9	1+
72	GFN	98	5.2	1-2+
48	GFN	79	5.3	1+
72	GFS	91	5.1	1-2+
48	GFS	122	5.2	1+

* C= Conventional; GF= Germfree; N= Bilateral Nephrectomy;
S= Sham Nephrectomy

Mean Blood Values

<u>Time Post-Op; Hrs.</u>	<u>Status*</u>	<u>BUN mgm%</u>	<u>Na, mEq/l serum</u>	<u>K, mEq/l serum</u>	<u>Ca, mEq/l</u>	<u>PO₄, mgm%</u>
72	CN	658	149	9.0	5.1	19.2
48	CN	313	160	4.6	7.2	12.9
72	CS	-	155	7.6	-	-
48	CS	27	142	4.4	6.0	7.2
72	GFN	399	160	4.9	8.0	32.6
48	GFN	285	166	4.9	9.4	22.1
72	GFS	27	154	5.0	5.6	10.0
48	GFS	33	164	5.5	7.8	9.0

* C= Conventional; GF= Germfree; N= Bilateral Nephrectomy;
S= Sham Nephrectomy.

Germfree Animals - Comparison of Serum Vs. Cecal-Contents Concentration

Time Post-Op. Hrs.	Status*	Urea N, mgm%		Na, mEq/l		K, mEq/l	
		Blood	Cecal	Serum	Cecal	Serum	Cecal
72	N	399	612	160	90	4.9	64.8
48	N	285	637	166	111	4.9	49.8
72	S	39	148	154	102	5.0	21.8
48	S	33	170	164	125	5.5	25.1

* N= Nephrectomized; S= Sham-nephrectomized.

Further chemical determinations are presently being carried out.

In summary, germfree rats are able to withstand uremia significantly longer than conventional rats. By contrast, germfree rats are not able to withstand starvation as well as conventional rats, nor are female rats able to withstand starvation as well as males. The lower tolerance for the lethal effects of starvation shown by germfree rats agrees with previous observations in this laboratory on germfree mice (Levenson and Tennant, Fed. Proc. 22:109, 1963). The biochemical response to uremia differs between germfree and conventional rats. The enlarged ceca of the germfree rats may play a significant role in the animal's response to uremia. Lesions were found in the cecum of conventional rats harboring Staphylococcus albus. These lesions resemble in many respects uremic enterocolitis in humans. These lesions were not found in germfree rats. Calcifications were found in the myocardium and aorta of germfree uremic rats. These were not present in conventional rats. The mechanisms of the difference in response to renal ablation between germfree and conventional rats are not entirely clear. Further studies are in progress to clarify these differences.

Effect of coprophagy in uremic conventional rats - rats are notorious for practicing coprophagy. The possible effects of the ingestion of feces containing various electrolytes, gastrointestinal secretions, products of bacterial metabolism, living bacteria, etc., upon the response of rodents to various disease states has not been clarified. We sought to determine the effect of coprophagy upon the response of rodents to bilateral nephrectomy. If this was found to be a significant factor, it would have to be controlled in future experiments on uremia in rodents.

Gustafsson and Anderson (PSEBM, 104:319, 1960), using a technique for preventing coprophagy in the rat, have described a significant reduction in anaerobic organisms in the feces of the rat. We sought to determine the effect of coprophagy on the course of uremia in open-room conventional rats.

The technique of Barnes et al. (Fed. Proc. 22:125, 1963) was used. Conventional male Wistar rats were allowed only 10% dextrose in 0.9% saline to drink 24 hours prior to surgery. Bilateral nephrectomy was carried out via laparotomy under aseptic conditions. Tail cups were then applied to all animals. The animals were allowed no food or water postoperatively. They were observed until death. Autopsy showed no significant lesions in either group.

The following results were obtained:

	Coprophagy Allowed (Sham-Tail Cups)	Coprophagy Prevented (Tail Cups)
No. Rats	8	8
Survival Time		
Mean	80.4 Hrs	85.2 Hrs
SE Mean	6.9 Hrs	2.9 Hrs

In summary, no statistically significant difference was found between uremic rats which were able to practice coprophagy and those prevented from doing so.

Liver Regeneration in Germfree and Conventional Animals.

Restoration of the size of the liver of the rat after surgical removal of approximately two thirds of the liver mass (the median and left lateral lobes) has been observed to occur in conventional rats after approximately two weeks. Surgical removal of less than two thirds of the liver results in a slower rate of restoration of the liver substance.

The stimulus for liver regeneration, and its cessation, has not been identified. It has, however, recently been linked to hepatic blood flow. The character of the portal blood differs between conventional animals, in which a metabolically active microbial flora is present in the gastrointestinal tract, and germfree animals, in which no microbial flora is present. It was thought that the pattern of liver regeneration in germfree rats might differ from that observed in conventional rats.

The method usually employed for the surgical removal of the liver is that described by Higgins and Anderson (Arch. Path. Lab. Med. 12:186, 1963). In this procedure, the median and left lateral lobes are resected and the assumption is made that 65-70% of the liver mass has been removed. To calculate the amount of liver regeneration which has occurred, the total liver weight must be estimated as a constant fraction of the total body weight and amount of liver removed must be estimated as a constant fraction of the total liver weight.

To test the validity of these assumptions, a series of rats were operated upon according to the procedure outlined, by Higgins and Anderson.

The following figures were obtained:

Partial Hepatectomy in Fischer Rats

Mean Values					
Status	No. & Sex	Body Wt., g	Total Liver, g	% Liver Removed	% Body Weight
Conv.	7 M	223	7.5	69	3.09
Conv.	7 F	150	4.3	68	3.20
Ex-GF	2 M	344	10.2	69	3.26
Ex-GF	3 F	217	7.1	69	3.26
GF	3 M	305	7.6	69	3.14
GF	2 F	198	4.3	68	2.16
GF	9 M	153	5.6	68	3.66
GF	9 M	153	3.8	67	2.49
Ex-GF	10 M	150	4.6	67	3.06

In spite of the fact that 52 rats were hepatectomized, there was considerable variation between the groups and greater variation among the individual animals.

These figures were used as a basis to estimate the regeneration of liver in two germfree animals. Two male Fischer rats were anesthetized

with Fluothane. A laparotomy was performed. Part of the liver was removed. The laparotomy was closed in two layers. The liver removed was weighed. Four days later, the rats were sacrificed, the liver removed and weighed. The following results were obtained:

Liver Removed	Regeneration
29%	177%
73%	102%

In summary, despite the variable statistics obtained in standardizing the procedure, it appears that germfree rats may regenerate lost liver tissue at a more rapid rate than conventional rats, and respond to a smaller amount of liver resection.

The variability of the fraction of the total body weight representing the liver weight and the fraction of liver removed place a severe restriction upon the interpretation of liver regeneration, however.

Plans are in progress for modifying a technique of tagging the liver parenchyma with Au¹⁹⁸ colloidal gold, as described by Lahr, *et al.* (J. Lab. & Clin. Med. 45:66, 1955), and adapting it to germfree work. This will allow calculation of the amount of liver regeneration by the dilution of the radioactive substance on an individual animal basis.

Spontaneous Tumors in Germfree Mice.

Pollard and Teah (J. Nat. Cancer Inst. 31:457, 1963) reported the spontaneous appearance of tumors in germfree rats, but not in germfree mice. Two "spontaneous" tumors have been noted in the course of doing routine autopsies on germfree ICR mice in this Department. These tumors were found in 9-month-old germfree mice, longer than most germfree mice are housed. One tumor was found in a male and one in a female. Two types of tumors were found, an adenoma confined to the lung, and a lymphoma which completely replaced the thymus and partially involved the liver, lung, and adrenal. The etiology of these tumors is not known. To our knowledge, this is the first report of the occurrence of "spontaneous" tumors in germfree mice.

Response of the Germfree Animal to Shock.

Bowel Shock: Germfree and Conventional(ized) Animals.

Limb Ischemia Shock: Germfree and Conventional(ized) Mice.

Burns: Germfree and Conventional(ized) Animals.

Radiation Injury.

(See Annual Progress Report: Role of Bacteria in Shock 3A012501B8130203, 1963-64).

Radiation Injury and Associated Biological Phenomena.

Supralethal whole-body x-irradiation.

Age versus radiation sensitivity.

Determination of LD₅₀ dose.

Radio-iodine studies (Thyroid activity).

Renewal of intestinal epithelium after x-irradiation. Studies with tritiated thymidine.

Trace metals by neutron activation analyses.

(See Annual Progress Report: Role of Bacteria in Shock 3A012501B8130203, 1963-64).

SUMMARY AND CONCLUSIONS:

Germfree, defined-flora, and conventional(ized) animals were examined in multidisciplinary studies to determine their constitution, physiology, and response to various stressors. The goals of these studies are to (1) define the host-parasite relationship, (2) determine the role and mechanisms of action of bacteria and their products, and (3) modify and regulate the latter's direct or indirect influence to the advantage of the host. The enlarged cecum of the germfree animal was studied with regard to its mode of filling, innervation, and content. Germfree and conventional animals were compared in: serotonin and histamine content of their tissues; tryptophane pyrrolase activity and its response to d- and l-tryptophane (and in uremia); morphological and humoral response of the lymphatic system to radiation; survival, histopathology, and biochemistry in uremia; response to antibiotics; response to parasitic infestation; and response to injury by limb ischemia, bowel ischemia, burns, and radiation. The process of conventionalization with bacteria was studied. Preliminary observations were made on inflammation, liver regeneration, and spontaneous tumors in germfree animals. Techniques were developed for anesthesia, gastrostomy,

and skin grafts to germfree isolators. Progress was made toward developing a colony of germfree hairless mice. Technological improvements were made on germfree and clinical isolators.

ARMY RESEARCH TASK REPORT				REPORTS CONTROL SYMBOL CSCRD-4(R2)	
ACCESSION NUMBER 36173			PROJECT, TASK, OR SUBTASK NO. 3A012501B8130801		
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D. C., 20315			2. FUNDING AGENCY Army Medical R&D Command Office of The Surgeon General Washington, D. C., 20315		
3. CONTRACTING AGENCY NA			4. CONTRACTOR AND/OR GOV'T LABORATORY A Walter Reed Army Inst of Rsch Walter Reed Army Medical Center Washington, D. C., 20012 723-1000, Ext 3552		
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Nauta, W.J.H., M.D., Ph.D., Dept of Neurophysiology Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012 576-2139 or Interdepartmental Code 198, Ext 2139 See Continuation Sheet					
6. TITLE OF: PROJECT <input type="checkbox"/> <div style="display: flex; justify-content: space-between;"> <div> TASK <input type="checkbox"/> SUBTASK <input checked="" type="checkbox"/> </div> <div> Analysis of behavior and of mediating mechanisms: Anatomic and electrophysiological factors (U) </div> </div>					
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964					
8. RESUME (U) Previous morphological and physiological studies of neural mediating mechanisms were extended. Several new projects were initiated. Morphological analysis of the mammalian and avian CNS was directed mainly toward the delineation of longitudinal systems interrelating successive levels of the central nervous organization. Physiological studies covered a wide range of neural mechanisms: a. The effects of temporal and spatial input characteristics upon single neuron responses in the brainstem auditory system; b. differential spontaneous and evoked activities of central nervous structures during sleep and wakefulness respectively; c. memory and read-out mechanisms exhibited by single neurons of the sea hare, <u>Aplysia californica</u> ; d. neural mechanisms involved in intracranial self-stimulation; e. regulatory mechanisms in hemorrhagic hypotension; f. central nervous control mechanisms involved in gastric secretion and adrenal cortical activity. Studies with direct clinical application were conducted, using improved methods for measuring quantitative changes in cerebral blood flow during anesthesia and hyperventilation, and for echo-encephalographic detection of pathological changes in cranio-cerebral topography. Several of the listed projects were accompanied and facilitated by new developments in technical instrumentation.					
9. KEY WORDS Neurology, neuropsychiatry, anatomy, physiology, behavior, circadian rhythms, krypton, circulation, stress response.					
10. SUPPORTING PROJECTS Not Applicable					
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <div style="display: flex; align-items: center;"> <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES </div>			12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T AGENCIES <div style="display: flex; align-items: center;"> <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES </div>		
See Continuation Sheet			See Continuation Sheet		
<div style="display: flex; justify-content: space-between;"> <div> DA FORM 1309R 1 June 63 </div> <div> PREVIOUS EDITIONS ARE OBSOLETE </div> <div> PAGE 1 of ____ </div> </div>					

ACCESSION NO.
36173

ARMY RESEARCH TASK REPORT

13. PROJECT, TASK OR
SUBTASK NUMBER

11 22 23 24 25 26 27 28 29
3 A 0 1 2 5 0 1 B 8 1 3 0 8 0 1

14. DATE OF REPORT (30-33)

30 33 34
0 6 6 4 4

15. SECURITY OF WORK (34)

16. TYPE OF REPORT

35 36 47 48 49 50 51 52 55
3 1 2 6 3

17. SCIENTIFIC FIELD

- a. Topical Classific. (56-61)
b. Functional Class (62-64)

56 61 62 64
0 1 0 6 0 5

18. OSD CLASSIFICATION
(65-66)

19. R&D CATEGORY (67)

65 66 67
B R 1

20. CONTRACT NUMBER

11 12 13 14 15 17 18 21 22 26 27
D A

21. GRANT NUMBER

28 29 30 33 34 35 36 38 39 40 41 45 46
D A G

22. ESTIMATED COMPLET.
DATES

47 51 52 56 57 61 62 66 67 71
1 C O N T 2 3 4 5

23. PRIORITY (11-14)

24. PROGRAM ELEMENT
(15-26)

11 14 15 26
2 6 • 1 1 • 2 5 • 0 1 • 1

25. CMR&D CODES

27 29 30 32 33 35
N / A

26. CDOG REFERENCE

- a. Paragraph No. (36-44)
b. Functional Group (45)

36 39 40 41 42 43 44 45
1 4 1 2 a 6

27. FUNDING

- a. Est. Total Cost (11-15)
b. % Spent Intern. (16-18)
" " Extern. (19-21)
c. Total Obligation (22-26)
d. Progrmd. Cur. FY (27-33)
e. " " " +1 (34-40)
f. " " " +2 (41-47)
g. " " " +3 (48-54)
h. " " " +4 (55-61)
i. " " " +5 (62-68)
j. " " " +6 (69-75)
k. Total Man Years of
Effort (76-78)

11 15 16 18 19 21 22 26
1 2
27 28 29 33 34 35 36 40
41 42 43 47 48 49 50 54
55 56 57 61 62 63 64 68
69 70 71 75 76 78

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

- (P) Heck, A.F., Capt, MC, Dept of Neurophysiology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-2139 or Interdepartmental Code 198, Ext 2139 49
- (P) Caggiano, V. J., Dept of Neurophysiology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-2021 or Interdepartmental Code 198, Ext 2021 49
- (P) McHenry, L. C., Capt, MC, Dept of Neurophysiology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-3610 or Interdepartmental Code 198, Ext 3610 49
- (P) Dreese, M. J., Capt, MC, Dept of Neurophysiology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-2516 or Interdepartmental Code 198, Ext 2516 49
- (P) Black, W. C., Capt, MSC, Dept of Experimental Psychology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-3031 or Interdepartmental Code 198, Ext 3031 49
- (A) Ebbesson, S.O., 1/Lt, MSC, Dept of Neurophysiology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-3460 or Interdepartmental Code 198, Ext 3460 49
- (A) Rupert, A., M.S., Dept of Neurophysiology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-3734 or Interdepartmental Code 198, Ext 3734 49
- (A) Kehoe, J., Ph.D., Dept of Experimental Psychology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D.C., 20012
576-3031 or Interdepartmental Code 198, Ext 3031 49
(NIH postdoctoral fellow)
- (A) Henson, C., Dept of Neurophysiology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-2139 or Interdepartmental Code 198, Ext 2139 49
- (A) Valenstein, E. S., Ph.D., Dept of Experimental Psychology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
(Presently with Fels Institute, Yellowsprings, Ohio) 33
- (A) Matthews, J., Dept of Experimental Psychology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-3031 or Interdepartmental Code 198, Ext 3031 49
(Under contract to University of Maryland)
- (A) Tolliver, G., Dept of Experimental Psychology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-3006 or Interdepartmental Code 198, Ext 3006 49

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

- (P) Cole, M., Capt, MC, Dept of Neurophysiology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-2139 or Interdepartmental Code 198, Ext 2139 49
- (P) Karten, H. J., M.D., Dept of Neurophysiology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-3460 or Interdepartmental Code 198, Ext 3460 49
(On contract to National Institutes of Health)
- (P) Petras, J. M., Ph.D., Dept of Neurophysiology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-2139 or Interdepartmental Code 198, Ext 2139 49
(National Institutes of Health postdoctoral fellow)
- (P) Astruc, J., M.D., Dept of Neurophysiology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-2139 or Interdepartmental Code 198, Ext 2139 49
(NIH foreign fellow from Spain)
- (P) Moushegian, G., Ph.D., Dept of Neurophysiology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-3734, or Interdepartmental Code 198, Ext 3734 49
- (P) Strumwasser, F., Ph.D., Dept of Neurophysiology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-3457 or Interdepartmental Code 198, Ext 3457 49
(Under grant from Washington School of Psychiatry)
- (P) Sheatz, G. C., Ph.D., Dept of Neurophysiology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-2139 or Interdepartmental Code 198, Ext 2139 49
- (P) Beer, Bernard, Ph.D., Dept of Experimental Psychology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-3031 or Interdepartmental Code 198, Ext 3031 49
- (P) Hodos, W., Ph.D., Dept of Experimental Psychology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-3031 or Interdepartmental Code 198, Ext 3031 49
(Under University of Maryland contract)
- (P) Smith, G., Capt., MC, Dept of Neuroendocrinology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-3027 or Interdepartmental Code 198, Ext 3027 49
- (P) McHugh, P. R., M.D., Dept of Neuroendocrinology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-3027 or Interdepartmental Code 198, Ext 3027 49
(Under Howard University contract)

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

- (A) Hall, V. R., Dept of Neurophysiology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-3009 or Interdepartmental Code 198, Ext 3009 49
(Under grant from Washington School of Psychiatry)
- (A) Swinnen, M. E. T., Dept of Neurophysiology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-3009 or Interdepartmental Code 198, Ext 3009 49
(Under grant from Washington School of Psychiatry)
- (A) Slocum, H. C., Col, MC, Dept of Surgery
WRGH, WRAMC, Washington, D. C., 20012
576-2381 or Interdepartmental Code 198, Ext 2381 49
- (A) Hayes, G. J., Col, MC, Dept of Surgery
WRGH, WRAMC, Washington, D. C., 20012
576-2381 or Interdepartmental Code 198, Ext 2381 49
- (A) Jenicek, J., Lt Col, MC, Dept of Surgery
WRGH, WRAMC, Washington, D. C., 20012
576-3380 or Interdepartmental Code 198, Ext 3380 49
- (A) Bivens, H. E., Capt, MC, Dept of Surgery
WRGH, WRAMC, Washington, D. C., 20012
576-3380 or Interdepartmental Code 198, Ext 3380 49
- (A) Mayes, H. A., Maj, MC, Dept of Surgery
WRGH, WRAMC, Washington, D. C., 20012
576-3379 or Interdepartmental Code 198, Ext 3379 49

REPORTS. Annual Progress Report, Walter Reed Army Institute of Research, 1 July 1963 - 30 June 1964.

McHenry, L.C., Jr.: Cerebral Blood Flow Determination in May by Kryton 85. Desaturation: a New Method. Nature, 200:1297-1299, 1963.

McHenry, L.C., Jr. and Levens, A.J.: The Treatment of Epilepsy with Drugs: A Resume. Dev. Med. & Child Neurol., 5:167-176, 1963.

Moushegian, G., Rupert A. and Whitcomb, M.A.: Medial Superior-Olivary-Unit Response Patterns to Monaural & Binaural Clicks. J. Acoust. Soc. Amer., 36: 196-202, 1964.

Sheatz, G.C.: An Inclined Plane Magnifying Ruler for EEG Y Axis Measurement. Electroenceph. Clin. Neurophysiol., 16:524-525, 1964.

Sheatz, G.C.: Distribution of EEG Activity Cycle Effects on Auditory Evoked Response. The Physiologist, 6: 274, 1963.

ACCESSION NUMBER

36173

ARMY RESEARCH TASK REPORT

Continuation Sheet

REPORTS, continued: 1. F. Strumwasser, J. J. Gilliam, and J. L. Smith: Long term studies on individual hibernating animals. Proc. Finn. Acad. Sci., Series A, 71/29, 1964.

2. F. Strumwasser, J. L. Smith, J. J. Gilliam, and F. R. Schlechte: Identification of active brain regions involved in the processes of hibernation. XVI International Congress of Zoology, 2:53, 1963.

3. F. Strumwasser, J. L. Smith, J. J. Gilliam, and F. R. Schlechte: Identification of active brain regions involved in the processes of hibernation. XVI International Congress of Zoology, 2:53, 1963.

DA FORM 1309R
1 June 63

PREVIOUS EDITIONS ARE OBSOLETE

Page of _____

ANNUAL PROGRESS REPORT

Project No. 3A012501B813 Army Medical Basic Research in Life Sciences.
Task No. 08 Neuropsychiatry
Subtask No. 01 Analysis of behavior and of mediating mechanisms: Anatomic and electrophysiological factors

Description: The general object of this subtask is the analysis of neural mechanisms mediating behavior in the widest sense. Within the frame of this general purpose, physiological studies are made of those mechanisms involved in 1. overt-behavioral and visceral-endocrine responses of the organism to organic and situational stress, to localized electrical stimulation of the central nervous system, and to ablations of various circumscribed parts of the brain; 2. the orientation of the organism in space and time, and, 3. diurnal and seasonal activity rhythms. Morphological studies are concerned mainly with the systematization of brain structures into longitudinal organizations on the basis of demonstrable interconnections between successive rostro-caudal levels.

Efferent connections of the neocortex. During the past year the main emphasis of this experimental-anatomical study has been on the subcortical projections arising from the precentral gyrus ("motor cortex" in the stricter sense of the term) and frontal contraversive eye field of the monkey. Particular attention was paid to the cortico-diencephalic projection. In the case of the precentral gyrus the latter involves the thalamic nuclei ventralis anterior and ventralis lateralis, reticularis, and centralis lateralis. The rostral subdivision of the gyrus (area 6 of Brodmann) has additional projections to the nuclei paracentralis, dorsomedialis, and parafascicularis; the caudal subdivision formed by Brodmann's area 4 projects to the nucleus ventralis posterior and centrum medianum. The frontal eye field was found to project to the thalamic nuclei reticularis, ventralis anterior, reuniens, paracentralis, centralis lateralis, dorsomedialis, and parafascicularis. Other projections of characteristic pattern were traced from both the precentral gyrus and frontal eye field to the subthalamus. Of special importance is an additional considerable projection of the frontal eye field to the superior colliculus.

Experimental-anatomical studies of the extrapyramidal motor system. The completed analysis of the striatofugal projection has been extended by an experimental study of projections from the substantia nigra. By a modified stereotaxic approach electrolytic lesions were placed in the substantia nigra of 6 cats and 1 monkey, the electrode entering the brainstem from the lateral side. By the use of the Nauta-Gygax silver technique for degenerating axons, fibers were traced to the thalamic nuclei ventralis medialis and ventralis anterior, to the superior colliculus, and to the midbrain tegmentum. Only few degenerating fibers could be followed to the globus pallidus and putamen, and none to the caudate nucleus. This latter negative

finding is incompatible with the marked cell degeneration appearing in the nigra following large caudate nucleus lesions. No explanation for this discrepancy can be offered at this time. The fiber degenerations traced to the thalamus appear to furnish acceptable evidence of a hitherto unknown nigro-thalamic connection. Those traced to the superior colliculus and midbrain tegmentum cannot be interpreted reliably, as they could represent corticofugal connections interrupted in their passage through the substantia nigra. Further experiments in chronically hemidecorticated monkeys, now in progress, are expected to settle this question.

Morphological studies of the submammalian central nervous system.

This project has been continued by an experimental-anatomical analysis of the cerebellofugal projections in the pigeon. The general purpose of the project is the elucidation of the hitherto largely obscure structural homologies between the submammalian and mammalian forebrain organizations. Such homologies are believed to be indicated most reliably by similarities of the neural in-and-out channels of the respective structures. The current study of the cerebellar projection has indeed begun to outline homologies in regard to certain structures in the medulla oblongata, pons, and midbrain. The avian diencephalon is much more difficult to compare to its mammalian counterpart. The present data need to be supplemented by information derived from experimental studies of other ascending systems distributing to the diencephalon. Such studies are planned, and in part already in progress.

A histological study of retrograde axon degeneration. The fate of the central segment of severed axons appears to have received little attention, and it is commonly held that it does not differ materially from the rapid Wallerian disintegration shown by the peripheral (i.e. amputated) part of the axon. The present study was initiated 2 years ago in an attempt to settle this question, the answer to which is of considerable importance for the interpretation of autoptical findings in case of chronic brain damage. The previous work on this subject has been continued in animals surviving high mesencephalic section of the medial lemniscus for periods of up to one year. Although the cell groups of origin of the medial lemniscus (contralateral nuclei gracilis and cuneatus) show heavy cell loss as a result of this surgery, the trajectory of the lemniscus proximal to the lesion shows no change other than a slow decrease of fiber caliber. A significant finding is that even after a 9 months survival massive Wallerian degeneration can be elicited in the atrophic bundle by lesion of the heavily "depopulated" nuclei gracilis and cuneatus from which it originates. The above-mentioned observations suggest that "retrograde fiber degeneration" is fundamentally different from the disintegrative process of anterograde (or Wallerian) degeneration. Unlike the latter, it is characterized by a slow fiber atrophy without drop-like disintegration of the fibers. The rate of atrophy in various stages of the process is currently being studied quantitatively by statistical sampling techniques.

Neuronal coding in the central auditory system.

a. Monaural and binaural tones. Within the auditory medulla there exists a class of neurons which when stimulated by binaural tones demonstrates a large latency shift. The range of this shift beyond the monaural latency varies from several milliseconds to 60 milliseconds depending upon the intensity of the sound in the ear opposite the driving ear. Also units in this part of the brain are responsive to changes in the binaural phase difference of low frequency sinusoids. These units are inhibited by in-phase binaural tones; however, as the phase angle is increased in small steps to 180° this inhibition is decreased. Beyond 180° , where now the opposite ear leads in phase, inhibition is effective again and at 330° the response grouping of the unit is identical to its response grouping at 30° . Monitoring of the cochlear microphonic responses at the two ears throughout these phase studies shows that no peripheral cancellations or distortions are taking place. Evidence for intrafiber volleying is shown by medullary units. These units discharge, during stimulation by monaural tones, with interspike intervals that are equal to and multiples of the period of the stimulating tone up to approximately 2.0 kc. This intrafiber volleying is maintained down to the unit's threshold at each of the effective frequencies.

b. Dichotic clicks. Our previous studies have shown that medial superior olivary (accessory nucleus) units of cat show a decrease in probability of response and an increase in latency when stimulated by binaural clicks of appropriate intensive and time differences. Recent findings from the same medullary region reveal a complex synaptic organization of neurons, some of which are differentially responsive to large and some to small interaural time differences. The former units which show binaural interactions to large time differences (ca. 10 msec.) are driven consistently by both monaural left and monaural right clicks. When, however, binaural clicks with large time disparities are delivered, the response probabilities to both clicks are significantly decreased. Some of the units which show binaural interaction to small time differences regularly fire twice to a monaural click. These units in contrast to the former ones, discharge only to one ear stimulation, never to both. To binaural clicks, either one of these spikes or both may be inhibited as time or intensive differences of the clicks are systematically changed. The spike latencies also of both responses are dependent upon these parameters of the clicks.

Mechanisms of innate pattern, learning, memory and read-out within single neurons. The model for this study is one of the large neurons (parabolic burster) in the parietovisceral ganglion of the gastropod, Aplysia californica. It is possible to isolate the ganglion and maintain its functional state in vitro under controlled conditions of sensory isolation, temperature, perfusion and mechanical stability for periods over two days. As shown by intracellular recordings this neuron exhibits a well-defined circadian and fortnight rhythm in its discharge frequency. Studies during the past year

have shown that the neuron in question can be conditioned by light-dark entrainment of the animal. When isolated from the rest of the organism, the neuron continues to show the particular circadian activity rhythm imposed upon it during the animal's conditioning period. Since there is good evidence that the activity of this cell is endogenous, this phenomenon must represent a read-out from "memory storage" within a single neuron.

Changes in evoked response amplitude and frequency related to the diurnal activity cycle. Studies of the influence of sleeping and waking on the evoked response were continued. A report was presented on 14 of the animals at the 1963 Fall meeting of physiologists at Coral Gables, Florida. Chronically implanted cats whose total sampled areas represent the major functionally identified surfaces and depths of the brain, are accustomed to the recording session and clicks and then responses are extracted from the sleeping and waking brain waves by the Mnemotron computer. The raw EEG is measured for frequency and amplitude and correlated with the evoked response. In most areas the evoked response tends to increase in sleep just as the spontaneous EEG invariably does. Frequency changes are less clearly related. An additional 12 cats have been studied but their recording areas have not been histologically verified. Efforts are in progress to search out and study more critically brain areas which do not increase the evoked response in sleep, the hope being that the two contrasting area groups will fall into some logical physiological pattern.

Intracranial self-stimulation.

a. Continuous opportunity for reinforcing brain stimulation. Because of the recent interest in brain stimulation as a possible reinforcer for prolonged studies of performance, and anecdotal reports of the unsatiable characteristic of reinforcing brain stimulation in some areas of the rat brain, a male albino rat weighing 444 g was placed into a 18x12x12 inch testing chamber in which a lever mounted on one wall provided the animal with a 0.5 sec train of bidirectional rectangular pulses of 0.4 ma for each lever respond. Based on previous experiences with this rat these stimulation parameters were judged to yield maximal response rates. Diluted condensed milk with Polyvisol was available at all times. During the next 20 stimulation days the total responses for ICS was 842,130. Daily response totals averaged 42,106 (range 25,811 - 93,712). The distribution of the non-responding time from day 3 through day 10 gave the following evidence of a diurnal rhythm: 17.7% of total non-responding time occurred between the hours of 0800 and 1200, 22.1% between 1200-1600, 21.1% between 1600-2000, 15.5% between 2000-2400, 12.6% between 2400-0400, and 10.6% between 0400-0800. Days 3 to 10 were selected for analysis as the animal's response rate during the first two days was significantly above average. This evidence for a persisting diurnal rhythm in self-stimulation performance is of special interest in view of the fact that the lights in the test chamber remained on during the entire experiment. The session was terminated before any evidence that the rat would have stopped responding if testing were continued. The animal was never observed to do anything but lever

press while awake, with the exception of brief "breaks" to eat and preen. Characteristically the animal slept directly under the lever and was observed to start lever pressing immediately upon awakening. At the end of the experiment the animal weighed 91% of its weight at the start of the experiment.

b. Rate of intracranial self-stimulation as a function of stimulus intensity and reinforcement density. The functional relationship between the intensity of intracranial stimulation (ICS) and the rate of self-stimulation in many areas of the rat brain increases at low intensities, reaches a maximum rate at "optimal" intensities and then declines at the higher intensities. This declining rate has been "explained" as a matter of spread of current away from positively rewarding cells to either neutral or aversive ones. Contrary to this position are data on preference tests which indicates that the intensity-preference function is monotonic, i.e. the higher the intensity the higher the preference for it. The present experiment was designed to analyze this paradox in terms of the temporal parameters of the reinforcement contingency. Three male albino rats were each stereotaxically implanted with a bipolar electrode aimed at the posterior hypothalamus. Following lever pressing training for brain stimulation reward a rate-intensity function was obtained for each rat. The schedule was continuous reinforcement (CRF) in which each lever response was reinforced by ICS. Three intensities were chosen for each rat. Low intensity was slightly above threshold for maintenance of self-stimulation behavior. Medium intensity was at the peak of the rate-intensity function. High intensity was twice medium and invariably resulted in a marked depression of the self-stimulation rate. The animals were then trained on a schedule in which lever responses were intermittently reinforced with ICS after an average variable interval of 10 seconds had elapsed since the previous reinforcement (VI 10 Sec). When this behavior had stabilized the animals were run daily allowing 9 five-minute trials to respond for the previously selected intensities. The CRF and VI 10 sec. schedules were alternated daily for 10 days. The procedure was then repeated comparing CRF with VI 20 sec. in one rat; VI 20, 30, and 45 sec. in the second rat; and VI 20, 30, 45, and 60 sec. in the third rat. In all CRF sessions the highest rate of responding came under the medium intensity and invariably an inverted "U" shape function was obtained. For each rat, it was possible eventually to generate a monotonic rate-intensity function by increasing the average interval of the VI schedule. One animal showed monotonicity at VI 10 sec., another at VI 45 sec and the third at VI 60 sec. An alternative to the notion of spread of current into negative areas as an explanation of the decline of self-stimulation rate at high intensities is that current spreads into areas which are principally motor. The resultant tremors, seizures and forced movements interfere with the smooth execution of the lever pressing response and the rate of responding declines. By using a schedule of intermittent reinforcement, the number of stimulations and consequently the effects of the motor disturbances are minimized over the course of time. Thus by reducing the reinforcement density, it has been possible to reconcile the rate-intensity function with the data obtained from intensity preference.

c. Adrenal system effects on electrical self-stimulation of the brain. A consistent and recent finding has shown that with electrical self-stimulation of hypothalamic and medial forebrain areas of the brain there have been correlated physiological changes similar to those changes found during stressful avoidance procedures. These effects include increased heart rate and blood pressure; elevated corticoids and catecholamines, and also depressed estrogens and androgens. Whether these changes are merely (1) effects of increased attention and general excitement in response to the brain stimulation, or (2) due to direct stimulation through current spreading to the nearby pituitary body, or perhaps (3) related to the reinforcing properties of the brain stimulus (i.e. these correlated changes are the reinforcing stimuli) is still a mystery. In an attempt to shed light on this problem a study was designed which would directly test these alternative explanations. The experimental subjects are male albino rats kept at 80% of their "ad lib" weight with continual free access to water. Bipolar stainless steel electrodes were implanted stereotaxically aimed at the posterior hypothalamus in nine animals and medial forebrain in nine others. On one wall of the experimental chamber is mounted two levers. The animals are trained to respond on one lever to receive food reinforcement and on the other for brain stimulation. Two auditory stimuli are then programmed which signal the proper condition of food or brain stimulation. Each condition is presented randomly and lasts for five minutes. After this base line training, one group of animals will receive various dosages of Bretylium or Guanethidine which will suppress sympathomimetic amines, also another group will have their pituitary body surgically removed while still a third group will have their adrenals removed. Each of these groups will include control animals with either placebo injections or sham surgery. The imposition of these experimental procedures will be tested against the baseline behavior for brain stimulation and food reinforcement.

Central nervous control of gastric secretion. This study has been conducted on rhesus monkeys equipped with chronic gastric and duodenal cannulae. Stimulation of deep brain loci was made possible by stereotaxic implantation of electrodes which were subsequently anchored to the skull by suitable mechanical devices. The animals were kept in restraining chairs enclosed in individual booths. Prior to stimulation through the indwelling intracranial electrodes, the monkey's gastric secretory response to acute aversive situations was determined, using the Sidman avoidance procedure in which the animal is required to press a lever in order to avoid a mildly painful electric shock to the foot. In early learning sessions, the animal's gastric secretion is inhibited while his plasma adrenocorticoid levels are increased. When subjected to a 72 hour avoidance period the animals first show a similar gastric inhibition which, however, gradually disappears during the second and third days. It was found that electrical stimulation of the amygdaloid complex or various points in the hypothalamus elicits the same pattern of gastric inhibition and adrenocortical activation that characterizes the avoidance response. The suggestion that the amygdala may be part of the neural mechanism involved in the gastric response to situational stress is being pursued further by studies of the gastric stress response shown by animals in which both amygdalae have been removed.

Central homeostatic control of adrenocortical activity. Previous studies of the central nervous regulation of adrenal cortical activity have demonstrated that a maximal increase in the plasma levels of adrenal steroids can be obtained by appropriately localized electrical stimulation of the hypothalamus. In further exploration of the brain-adrenal cortex relationship it was found that a marked, although somewhat lesser increase in adrenal steroid production can also be elicited by stimulation of the amygdaloid complex, a structure known to be connected to the hypothalamus by well-developed neural pathways. Studies in the monkey performed during the past year have shown 1. that the adrenocortical response to amygdaloid stimulation is contingent upon the production of neural afterdischarge activity in the amygdaloid complex, and, 2. that the magnitude of the response depends on the level of adrenal steroids already present in the blood plasma. After pre-loading with exogenous corticosteroid to produce plasma levels corresponding to the highest levels observed during amygdaloid stimulation, no further increase of the plasma level can be obtained by stimulation of the amygdaloid complex, although hypothalamic stimulation can still produce a further rise under these conditions. This observation suggests the existence of a negative 'external feedback' mechanism mediated by the corticosteroids proper, and regulating the extent to which neural impulses of amygdaloid origin can succeed in activating the hypothalamic neurons involved in the release of ACTH by the anterior pituitary.

Neural regulatory mechanisms in hemorrhagic hypotension. Studies of acute blood loss in dogs were continued and some effects of various anesthetics on phenomena of the pulse rate and blood pressure were noted. A patterned response of the pulse rate to blood loss was determined. Temporal characteristics of this pattern as well as magnitudes, changes and rates of change in pulse rate, blood pressure and blood volume were subjected to a nonparametric statistical analysis (Spearman coefficient) utilizing the RPC 400 computer (WRAIR). This analysis is presently being evaluated for the significance of direct and inverse correlations found between the parameters measured. The prototype for a miniaturized transducer for use in a new technique for rapid serial qualitative and semiquantitative measurement of venous return to the heart has been built. A new technique employing fiber optics to obtain continuous differential plethysmographic readings from the brain and skin of experimental animals was developed. An electronic monitoring system for accurate measurement of heart rate and pulse wave velocities (propagation times) was designed and built. Some of the physiological characteristics of these recordings were documented both acutely (4-8 hours in anesthetized cats) and chronically (30-70 days in rhesus monkeys) by noting the effect of certain drugs and maneuvers on these parameters. Physiologic reactance and tissue tolerance for the technique were evaluated through detailed pathoanatomical studies on the brains of these animals. Experiments dealing with the problems of implantation of the new device into liver, gut and muscle have been initiated for the purpose of providing a model for study of simultaneous vasomotor changes in these various organs during blood loss, hypotension and other states of physiologic alteration.

Clinical-physiological and echo-encephalographical studies. Studies of alterations in cerebral volume by measuring quantitative changes in cerebral blood flow during anesthesia and hyperventilation were continued from last year. In addition changes in cerebral volume and pathological damage to the brain were studied with rheoencephalography. Echoencephalography (Echo EG), a new tool for clinical and research evaluation of alterations in cranio-cerebral topography, particularly shifts in the intracranial mid-line, was utilized in studies of head trauma and other brain disease.

The clinical and research use of echoencephalography (Echo EG) in the evaluation of midline shifts and alterations of the lateral structures of the brain. During the period from August 1963 to the present, Echo EG examinations have been carried out on 363 patients. Whenever possible, results of other neurological studies (i.e. EEG, arteriography, pneumoencephalography, rheoencephalography) and necropsy findings were correlated with Echo EG results. Experience has allowed Echo EG to be used for more than simple midline determination, for example following the course of cerebral swelling, and recognition of the significance of lateral echos. The dynamic changes in intracerebral relationships resulting from the pulsatile influx of blood, have been recorded cinematographically. Pulsations on the echogram synchronous with the heart were found to arise from an intracerebral aneurysm.

The effects of hyperventilation on the cerebral circulation and metabolism. These studies were carried out to determine the effects of hyperventilation on cerebral metabolism, and to establish whether or not moderate respiratory alkalosis will produce ischemic cerebral anoxia. Cerebral blood flow studies were performed by the krypton 85 desaturation technique before and during hyperventilation in 8 awake subjects and in 8 subjects during halothane anesthesia prior to elective general surgery. Halothane anesthesia alone caused an increase in cerebral blood flow (CBF). Hyperventilation produced a diminution in CBF, but no change in cerebral oxygen consumption ($CMRO_2$) in either awake or anesthetized individuals. Although there was no fall in the cerebral metabolic rate, there was a decrease in the jugular venous oxygen content with hyperventilation which in some ways parallels the fall in cortical oxygen tension. In moderate respiratory alkalosis (i.e. pCO_2 22.7 mm Hg; pH 7.54) the mean jugular oxygen content was 7.66 vol. % and no value reached a critical level of less than 4 vol. % (Lennox, 1935). Moderate hyperventilation therefore does not appear to produce ischemic cerebral anoxia and has been shown to be of benefit during craniotomy under halothane anesthesia where the vasoconstrictive effects of hypocapnia will overcome the vasodilatory effects of halothane to prevent an increase in cerebral blood flow and brain volume.

The use of rheoencephalography (REG) in monitoring changes in intracranial pressure and in the evaluation of disturbance in cerebral circulation. Rheoencephalograms have been performed in 130 individuals. A study of 20 normal individuals, 10 with simultaneous cerebral blood flow studies, was performed to determine the range of normal wave form and values. Considerable variation in the REG wave form was found in normal individuals particularly in the height of

the wave, measured in milliohms resistance, and the form and degree of angle of decline. A secondary peak, corresponding with that on the arterial pulse wave, was usually present. Rarely there were one or two small waves, one of which corresponded to the arterial pulse wave. The primary and secondary REG waves probably reflect the changes in intracranial pulse volume that occur with arterial pulsation in the brain, with a minor contribution from the extracranial circulation. In a study of 100 patients with pathological changes in the brain two observations have been made in a preliminary evaluation of the data. One, in patients with diminished cerebral circulation due to cerebral vascular disease with clinical manifestations there is almost invariably an alteration in the form of the REG wave, a decreased angle of inclination and usually an increase in the EKG "R" wave to REG peak interval. The changes in the REG wave show considerable variation from patient to patient and no definite criteria can be established. Two, increased intracranial pressure produces a characteristic rounding or upward convexity to the REG wave.

Technical developments.

a. Neuron counts in the nervous system. The number of nerve cells in a given section is most reliably determined by counting the nucleoli. Despite the small size of the nucleolus, a source of considerable inaccuracy is presented by the presence of bisected nucleoli, one portion of which is counted in one section and the other part in the adjacent section. Many previous investigators have employed correction formulas to compensate for this error, but in all these correction procedures insufficient attention has been paid to 1) the magnification at which the particles are counted, and 2) the role of section thickness. By the use of hypothetical models as well as of actual counts of nuclei and nucleoli, an improved counting procedure was developed in which nucleoli are counted in pairs of adjacent sections of unequal but rigorously controlled thickness (e.g. 6 and 15 μ). The actual number of nucleoli N in either section is then equal to the product of the section thickness and the difference between the particle counts obtained in the two adjacent and unequal sections, divided by the difference of the section thickness.

b. Experimental-anatomical staining methods. The technique for counterstaining of Golgi-stained material described in the previous Annual Report appeared in print in the journal Stain Technology. No further major advances can be reported at this time. Work in this field has been continued with the specific purpose of developing a more suitable technique of selective Silver impregnation of degenerating synaptic boutons in the central nervous system. The need for such a method is evident and will steadily increase as the general distribution areas of more and more fiber systems become known. Findings made during the past year suggest that the physico-chemical properties of degenerating boutons may be sufficiently different from the norm to allow the development of selective impregnation techniques. Thus far, the use of pre-chromation of tissue sections has yielded the most promising results, but much work remains to be done before a consistently effective method can be formulated.

c. Fiber optics plethysmography. A new technique employing fiber optics was developed, by which continuous plethysmographic readings from deep-seated organs such as the brain or from the skin can be obtained. A detailed description of this method has been accepted for publication by the Journal of Applied Physiology.

Electronic programming, recording and analyzing devices. During the past year several new instruments have been developed in the biomedical engineering laboratory: 1) an on-line system for recording physiologic time intervals. It accurately measures and records in digital form time intervals between pulsatile physiologic events in the cardiovascular system; 2) a versatile programmer for ratio and interval schedules used in behavioral research studies. To train experimental animals by means of progressive or other ratio programs this highly versatile programmer makes use of a prepunched tape in order to control the entire operation, allowing automatic advancing, resetting when required, warning the operator when to intervene manually, supplying reinforcements, homing, and starting or stopping the program at predetermined times or coincident with occurring events. 3) a dual-channel on-line E.M.G. integrator. This device enables a physiologist to make long-term comparisons between the activity at two separate E.M.G. electrodes. The data are obtained in digital form. 4) a low-level counter and pulse shaper. This instrument was made for the purpose of counting the number of potential spikes per unit time originating from the firing of single brain cells. It will, however, count and transform any non-symmetrical wave of short duration and low amplitude to an electrical pulse of suitable form. Two separate versions of this device have been in use in the Department of Neurophysiology. 5) an automated brain electrode impedance measuring device. A six-channel printing recorder scans and prints data every 2 seconds from 6 different sources. In this particular instrument two channels record the accumulated number of lever pressure of the experimental animal, two channels measure the electrical impedance of an implanted pair of brain electrodes and two channels measure the impedance of two different precision resistors of known value. This last measurement constitutes a constant self-check of the machine. 6) a coding device for a tape recorder. As part of an automated teaching machine a binary coder has been developed which modulates and demodulates up to five signals (binary 32) on a two-track tape. The instrument provides dry contacts to operate accessory equipment. 7) current regulated electrical stimulator. This stimulator is used by several investigators engaged in various aspects of nervous system research. The stimulator provides current regulated pulses or voltage regulated spikes precisely controlled over a wide dynamic range. Both voltage and current monitors are provided, isolated from the actual output which is also isolated and floating. This stimulator also provides a completely balanced output which minimizes brain tissue damage. The stimulator is extremely versatile and can be used in virtually any application requiring an electrical stimulus without the use of additional timing equipment. 8) histogram. An on-line data processor was constructed capable of obtaining, recording and analyzing data from living neuron specimens. The system was used in studies of the neural mechanisms of auditory discrimination. The system was assembled from commercial plug-in printed circuit cards with patch board capability.

for changing the program as new requirements appear. At present, the system is capable of measuring frequency (events per unit of time), latency, and waveform interval analysis (histogram). The histogram at present has twenty channels, each channel capable of holding maximum number of six digits. The "basket widths" (histogram intervals) available are .1, .2, 1, 2, 10, 20, 100, 200, 1000 ms. Readout may be obtained in analog or digital form. Any one of the twenty channel contents can be presented in an analog form or may be printed out in digital form with a Hewlett-Packard recorder. The contents of each channel can also be displayed with nixie readout tubes. 9) an automated brain electrode impedance measuring device. One of the variables rarely measured in studies involving electrical stimulation of the brain is the impedance change of the neural area directly under the tips of the stimulating electrodes. Information concerning the relationship between electrical brain stimulation and impedance changes across the electrode may account for a great deal of variability in the data obtained from brain stimulation experiments as well as helping to build reliable and non-injurious stimulating devices. With these problems in mind an apparatus was designed which would measure impedance between two electrodes implanted in the brain while concurrently integrating and recording electrical stimulation presented through the same pair of electrodes. The impedance would be measured with respect to 100 cps and 1000 cps with maximum allowable voltage for measurement arbitrarily set at 50 mV. The system was built around a Leeds and Northrup, Speedomax, 6-channel recorder. The full scale sensitivity is 1 mV. and it scans and prints for 2 seconds per channel. A microswitch was added to the stepping mechanism of the recorder to obtain a synchronizing pulse each time a new channel is being scanned. This pulse controls a six position electro-mechanical stepper which connects the required input to each channel and inhibiting the others at the same time.

Summary and Conclusions:

Previous morphological and physiological studies of neural mediating mechanisms were extended. Several new projects were initiated. Morphological analysis of the mammalian and avian CNS was directed mainly toward the delineation of longitudinal systems interrelating successive levels of the central nervous organization. Physiological studies covered a wide range of neural mechanisms: a. The effects of temporal and spatial input characteristics upon single neuron responses in the brainstem auditory system; b. differential spontaneous and evoked activities of central nervous structures during sleep and wakefulness respectively; c. memory and readout mechanisms exhibited by single neurons of the sea hare, Aplysia californica; d. neural mechanisms involved in intracranial self-stimulation; e. regulatory mechanisms in hemorrhagic hypotension; f. central nervous control, mechanisms involved in gastric secretion and adrenal cortical activity. Studies with direct clinical application were conducted using improved methods for measuring quantitative changes in cerebral blood flow during anesthesia and hyperventilation, and for echo-encephalographic detection of pathological changes in cranio-cerebral topography. Several of the listed projects were accompanied and facilitated by new developments in technical instrumentation.

ARMY RESEARCH TASK REPORT		REPORTS CONTROL SYMBOL CSCRD-4(R2)
ACCESSION NUMBER 36174	PROJECT, TASK, OR SUBTASK NO. 3A012501B8130802	
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D.C. 20315	2. FUNDING AGENCY Army Medical R&D Command Office of The Surgeon General Washington, D.C. 20315	
3. CONTRACTING AGENCY NA	4. CONTRACTOR AND/OR GOV'T LABORATORY Walter Reed Army Inst of Rsch Walter Reed Army Medical Center Washington, D.C. 20012 723-1000, Ext 3552	
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Brady, J.V., Lt Col, MSC, Division of Neuropsychiatry, Dept of Experimental Psychology, WRAIR, WRAMC, Washington, D.C. 20012 576-2483 or Interdepartmental Code 198, Ext 2483. See Cont Sheet		
6. TITLE OF: PROJECT <input type="checkbox"/> TASK <input type="checkbox"/> Analysis of behavior and of mediating mechanisms: SUBTASK <input checked="" type="checkbox"/> Experimental psychological factors (U)		
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964		
8. RESUME (U) The basic program can be described under three types of projects. 1) The experimental analysis of complex behavioral repertoires and the study of basic variables affecting behavior. 2) Behavioral methods for producing stress and fatigue. 3) The effects of stress-inducing procedures on complex behavioral repertoires. A number of complex repertoires have been studied. Timing behavior, acquisition of new behavioral chains, acquisition of new stimulus discriminations, collateral behavior during temporal discriminations, schedule preference, adjusting avoidance behavior, vigilance, matching-to-sample, and basic social behavior have received experimental attention. The production of stress and fatigue has focused upon prolonged sessions of avoidance conditioning where the animal must continue to work for extended time periods in order to avoid an electric shock. The chronic effects of stress were studied by forcing monkeys to undergo repeated 72-hour avoidance sessions over extended periods greater than a year. In addition to behavioral studies, observations were made of changes in patterns of thyroid, gonadal, and adrenal hormone secretion. The behavioral effects of stress and fatigue have been evaluated primarily in terms of timing behavior and by decrements in performances established by Sidman avoidance, paced avoidance, adjusting avoidance, and conditioned fear techniques.		
9. KEY WORDS Behavior, experimental psychology, stress, fatigue, behavioral repertoires, operant behavior.		
10. SUPPORTING PROJECTS Not Applicable		
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet	12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet	
DA FORM 1309R 1 Jun 63 PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of 1		

ACCESSION NO.

36174

ARMY RESEARCH TASK REPORT

13. PROJECT, TASK OR
SUBTASK NUMBER

11	22	23 24	25 26	27 28	29
3 A 0 1 2 5 0 1 B 8 1 3	0 8	0 2			

14. DATE OF REPORT (30-33)

30	33	34
0 6 6 4	4	

15. SECURITY OF WORK (34)

16. TYPE OF REPORT

35	36	47 48	49 50	51	52	55
3					1 2 6 3	

17. SCIENTIFIC FIELD

 a. Topical Classific. (56-61)
 b. Functional Class (62-64)

56	61	62	64
0 1 0 6 0 5			

18. OSD CLASSIFICATION
(65-66)

19. R&D CATEGORY (67)

65 66	67
B R	1

20. CONTRACT NUMBER

11 12	13 14	15	17	18	21	22	26	27
D A								

21. GRANT NUMBER

28 29	30	33	34 35	36	38	39 40	41	45	46
D A							G		

22. ESTIMATED COMPLET.
DATES

47	51	52	56	57	61	62	66	67	71
1 C O N T	2			3		4		5	

23. PRIORITY (11-14)

24. PROGRAM ELEMENT
(15-26)

11	14	15	26
	2	6 • 1 1 • 2 5 • 0 1 • 1	

25. CMR&D CODES

27	29	30	32	33	35
N / A					

26. CDOG REFERENCE

 a. Paragraph No. (36-44)
 b. Functional Group (45)

36	39	40	41 42	43 44	45
1 4 1 2			a		6

27. FUNDING

- a. Est. Total Cost (11-15)
-
- b. % Spent Intern. (16-18)
-
- " " Extern. (19-21)
-
- c. Total Obligation (22-26)
-
- d. Progm. Cur. FY (27-33)
-
- e. " " " +1 (34-40)
-
- f. " " " +2 (41-47)
-
- g. " " " +3 (48-54)
-
- h. " " " +4 (55-61)
-
- i. " " " +5 (62-68)
-
- j. " " " +6 (69-75)
-
- k. Total Man Years of
-
- Effort (76-78)

11	15	16	18	19	21	22	26
		1		2			
27 28	29	33	34 35	36	40		
41 42	43	47	48 49	50	54		
55 56	57	61	62 63	64	68		
69 70	71	75	76	78			

DA FORM
1 June 63 1309R

Previous Editions are Obsolete

Page 2 of _____

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

- (P) Boren, J.J., Ph.D., Dept of Experimental Psychology, Division of Neuropsychiatry, WRAIR, WRAMC, Washington, D.C. 20012
576-3007 or Interdepartmental Code 198, Ext 3007 49
- (P) Hodos, Wm., Capt, MSC, Ph.D., Department of Experimental Psychology, Division of Neuropsychiatry, WRAIR, WRAMC, Washington, D.C. 20012
576-2483 or Interdepartmental Code 198, Ext 2483 49
- (P) Beer, B., M.A., Department of Experimental Psychology, Division of Neuropsychiatry, WRAIR, WRAMC, Washington, D.C. 20012
576-3031 or Interdepartmental Code 198, Ext 3031 49
- (P) Kehoe, JacSue, Ph.D., USPHS Post-Doctoral Fellow, Department of Experimental Psychology, Division of Neuropsychiatry, WRAIR, WRAMC, Washington, D.C. 20012
576-3031 or Interdepartmental Code 198, Ext 3031 49
- (P) Black, Wm.C., Capt, MSC, Ph.D., Department of Experimental Psychology, Division of Neuropsychiatry, WRAIR, WRAMC, Washington, D.C. 20012
576-3031 or Interdepartmental Code 198, Ext 3031 49
- (P) Mason, J.W., M.D., Dept of Neuroendocrinology, Division of Neuropsychiatry, WRAIR, WRAMC, Washington, D.C. 20012
576-3559 or Interdepartmental Code 198, Ext 3559 49
- (A) Tolliver, G.A., B.A., Department of Experimental Psychology, Division of Neuropsychiatry, WRAIR, WRAMC, Washington, D.C. 20012
576-3008 or Interdepartmental Code 198, Ext 3008 49
- (A) Keller, Barbara K., M.A., Department of Experimental Psychology, Division of Neuropsychiatry, WRAIR, WRAMC, Washington, D.C. 20012
576-5262 or Interdepartmental Code 198, Ext 5262 49
- (A) Devine, D.D., SP/4, U.S. Army, B.A., Department of Experimental Psychology, Division of Neuropsychiatry, WRAIR, WRAMC, Washington, D.C. 20012
576-3007 or Interdepartmental Code 198, Ext 3007 49
- (A) Kilminster, A., Pfc, U.S. Army, B.A., Department of Experimental Psychology, Division of Neuropsychiatry, WRAIR, WRAMC, Washington, D.C. 20012
576-3008 or Interdepartmental Code 198, Ext 3008 49

REPORTS. Annual Progress Report, Walter Reed Army Institute of Research, 1 July 1963 - 30 June 1964.

Brady, J.V. Behavioral stress and physiological change: A comparative approach to the experimental analysis of some psychosomatic problems. Trans. N.Y. Acad. Sci., Series II, 26:5,483-496, Feb 1964.

Brady, J.V. Further comments on the gastrointestinal system and avoidance behavior. Psychol. Rep., 12:742, 1963.

Field, G.E. and Boren, J.J. An adjusting avoidance procedure with multiple auditory and visual warning stimuli. J. exp. Anal. Behav., 6:537-543, 1963.

Hodos, W. A reinforcer for cats. J. exp. Anal. Behav., 6:162, 1963.

Hodos, W. A simple method for the description of inter-response time distributions. J. exp. Anal. Behav., 6:90, 1963.

Migler, B. Bar holding during escape conditioning. J. exp. Anal. Behav., 6:65-72, 1963.

Migler, B. Experimental self-punishment and superstitious escape behavior. J. exp. Anal. Behav., 6:371-385, 1963.

Migler, B., and Brady, J.V. Time behavior and conditioned fear. J. exp. Anal. Behav., 7:247-251, 1964.

Perez-Cruet, J., Black, W.C. and Brady, J.V. Heart rate: Differential effects of hypothalamic and septal self-stimulation. Science, 140:1235-1236, 1963.

Perez-Cruet, J., Tolliver, G., Dunn, G., Marvin, S. and Brady, J.V. Concurrent measurement of heart rate and instrumental avoidance behavior in the rhesus monkey. J. exp. Anal. Behav., 6:61-64, 1963.

Valenstein, E.S., and Beer, B. Continuous opportunity for reinforcing brain stimulation. J. exp. Anal. Behav., 7:183-184, 1964.

ANNUAL PROGRESS REPORT

Project No. 3A012501B813

Title: ARMY MEDICAL BASIC RESEARCH
IN LIFE SCIENCES

Task No. 08

Title: Neuropsychiatry

Subtask No. 02

Title: Analysis of behavior and of
mediating mechanisms: Experimental psychological factors

Description: The scientific investigations to be described below have encompassed three primary aims. 1) to establish and to study complex repertoires of behavior, including behaviors of basic scientific interest, 2) to explore methodology for producing stress and fatigue, 3) to analyze the effects of stress and fatigue by the disruption of complex behavioral repertoires.

The studies on behavioral repertoires include the topics of timing behavior, schedule preference, behavioral performances and retention of stimulus discriminations during hibernation, stimulus generalization, behavioral variability, chronic extinction behavior, general activity, acquisition of new behavioral chains and of stimulus discriminations, adjusting avoidance, vigilance, matching-to-sample, and social interactions among primates. The production of stress and fatigue has been accomplished primarily by submitting animals to prolonged sessions of avoidance conditioning. Investigations have been made of changes in behavior and hormonal patterns due to repeated 72-hour sessions of Sidman avoidance, and of deteriorated timing behavior produced by paced avoidance, and by conditioned fear.

Progress: Several experiments have focused on the role of mediating responses in temporal discrimination. A chain of responses which fills in the temporal "gap" could serve to mediate the discrimination of the passage of time. Recently, these collateral responses have been recorded in rats, monkeys, and humans. The next step in the experimental analysis of collateral responding is to determine its relationship to specific aspects of the temporal discrimination.

In the first experiment, a monkey was required to demonstrate a temporal discrimination by pressing one lever three times and then after a specified time delay pressing a second lever. If the required period of time had elapsed from the first response on the first lever to the response on the second lever, the monkey was rewarded with food pellets. If he failed to meet the requirements of three responses on the first lever and a minimum delay between levers, he received no reward. The delays used were 3, 4, 7.5, 15, 30, 45 and 60 seconds. The behavior

of the monkey adjusted well to each successive increase in the delay requirements, thus indicating that the animal was performing the temporal discrimination. However, the number of responses on the first lever increased from a median value of 4 on the 3 second delay to a median value of 26 on the 45 second delay. Since the requirement of 3 responses on the first lever was unchanged during the course of the experiment, the sizeable increase in the number of responses above the required level as a function of the delay interval suggests these additional responses may function as a response chain which "superstitiously" satisfies the temporal requirements of the experiment. The experiment has been repeated in a descending order of delay times with essentially the same results.

A second experiment was carried out to determine whether repetitive exteroceptive stimuli could also function as mediators of temporal discrimination. A cat was trained to perform the same type of two-lever temporal discrimination as the monkey in the previous experiment. However, in this case, only one response was required on the first lever. If at least 18 seconds elapsed between a response on the first lever and a response on the second, the cat was rewarded with a small quantity of fish. Every two seconds during the interval between the two responses a 2.0 second tone was presented. Intermittently, the duration of the tone was changed to 1.9 seconds or 2.1 seconds. On those test trials in which the tone was shorter than normal, the cat consistently underestimated the 18 second interval. On those test trials in which the tone was longer than usual, the cat consistently overestimated the 18 second interval. These findings indicate that the cat, at least, is capable of using a repetitive stimulus to mediate a temporal discrimination.

In a third experiment, animals were trained to push a disc for a food reward. When the disc was red, the subjects were required to push the disc 100 times in order to receive the reward. When the disc was green, the animals were rewarded only after a period of 2 minutes had elapsed since the previous reward. During this condition, the subjects increased their response rate as the 2 minute interval drew to a close. This indicated the development of a type of temporal discrimination. When the disc was orange, the subjects were required to delay their responses by an interval of at least 10 seconds. Responses which were emitted less than 10 seconds following the previous response were unrewarded. Those responses which were delayed for the required time period were reinforced with food. In this condition, the animals slowed their rate of responding, thereby indicating the development of another type of temporal discrimination. Finally, when the disc was white, pushing the disc was never followed by a reward. In addition to recording the responses of the animals, data were also obtained from a set of micro-switches mounted under the movable floor of the experimental chamber. These switches were closed each time the subjects walked, jumped, or moved. The data obtained from the floor switches indicated clearly that, during each of the experimental conditions, the subjects were

emitting a considerable number of responses other than pushing the disc. Moreover, these responses, which consisted of pacing, jumping, and moving, had a systematic relationship to the various conditions of the experiment. During those conditions in which time was a critical variable in obtaining reward, the animals emitted a far greater number of collateral responses than they did during the condition in which only the number of responses determined the delivery of the food. These data add further support to the notion that organisms rely on chains of responses to mediate the passage of time. They also emphasize the value of examining the total behavior of the organisms under investigation.

Another experiment investigated timing behavior as a function of the amount of reinforcement. Either one, two or four food pellets were presented whenever an animal spaced his responses longer than 18 seconds, while responses shorter than 18 seconds reset the trial. The "timing" behavior generated depended on the amount of reinforcement. "Efficiency" ratios (reinforced responses/total responses) were highest for the one-pellet condition and lowest for the four-pellet condition. The mode of inter-response time distributions during the four-pellet condition revealed shorter response times while the one-pellet condition showed a less peaked and more variable distribution.

An experiment still in progress is concerned with the effects of food deprivation on preference for different types of response chains reinforced by food. The animal is faced with a time-dependent chain and a chain for which timing is not a prerequisite for reinforcement. Under the response dependent chain (a fixed ratio schedule) increasing the deprivation level tends to increase efficiency, but under the time-dependent chain, efficiency typically decreased with increased deprivation. This experiment has been measuring the effects of deprivation on the animal's choice between a constant valued DRL schedule (one in which the animal must separate his responses by 15 seconds) and a set of five fixed ratio schedules. Although the data are not quite complete, the rats seem to prefer the ratio schedules, and take even the higher and more demanding ratios over the time-dependent chain when deprivation increases. One would predict this result from the efficiency of FR performances seen under higher deprivation levels when the two different types of chains are studied independently.

Another study of preference is investigating the problem of whether an increased work load increases the value of an obtained reinforcement. With the monkey as the experimental subject, a procedure has been devised whereby various amounts of work precede a preference measure for a one gram food pellet. The work loads are in the form of a fixed number of lever responses (FR) on lever 1 in the presence of a light over lever 1. When the FR requirements are met, the lever 1 light goes out and the light over lever 2 comes on. During this condition

the animal must respond on lever 2 after a fixed interval of four minutes (FI 4 min) for which he receives a food pellet. The rate of lever 2 responses during the FI 4 min has been shown to be a sensitive indicator of incentive preference.

Brain temperature and food and water intake were measured on a *Citellus lateralis* ground squirrel during the last half of his hibernating season. In spite of a constant cold environment, this squirrel shows periodic rather than continuous hibernation. The experiment indicated that the animal could and did perform responses to obtain food and water each of the nine times he aroused from hibernation. However, his food intake was negligible until the last two arousal periods prior to the end of the season. The experiment also indicates that the animal moves a great deal during these arousals, and that he is quite capable of remembering a previously learned response, in spite of the prolonged hypothermic state that has intervened since its acquisition.

Eight *Citellus lateralis* ground squirrels were trained on a visual discrimination in which they were reinforced for selecting a nose key illuminated with constant rather than flickering light. Four of these animals spent 30 days of their retention interval in a cold environment of approximately 5° centigrade, and next temperatures indicated that three of these animals hibernated during part of this month. The other four animals were maintained at normal room temperature during this period. For 13 additional days, the animals were kept in their home cages until they returned to their experimental weights. All eight animals were tested after this 43 day period in the experimental apparatus, to see if there was any effect of the hibernation on the performance of the visual discrimination. The decrement in performance shown by the groups was almost identical, approximately 16% for both groups. Thus, hibernation and its physiological changes seem neither to help nor to hinder the retention of a previously acquired visual discrimination.

In an experiment dealing with stimulus generalization, rats were trained to press two keys consecutively for a food reinforcement. During stimulus #1 (slow click rate) a 6 second time delay was required between the two responses and during stimulus #8 (fast click rate) no time delay was necessary. When tested with intermediate stimuli (medium click rates), the rats seemed to delay for intermediate times between the two responses. This interpretation was based on median and averaged data. Closer examination of the delay data, however, revealed that the median and average data were not representative of a central tendency but rather were artifacts of averaging bimodal distributions. An animal's performance in any particular intermediate stimulus was to respond with zero delay or with a 6 second delay. The exact mixture of the two delays (and therefore the average delay) depended on the similarity of the test stimulus to the training stimuli.

Experiments with rats, pigeons, and children have shown that reinforcement schedules influence behavioral plasticity in a multi-choice situation. The present research corroborates previous rat and child findings and further demonstrates that the age of the organism is a critical variable. Single lever pretraining on continuous, fixed and variable ratio differentially influences behavior when the organism is given access to four levers simultaneously, responses on any one of which is reinforced on continuous reinforcement. Continuous and fixed ratio pretraining generate a preponderance of responses on one or two levers, while variable ratio pretraining generates an even distribution over the four keys. Intermittent schedules generate greater variability in young rats (less than 90 days of age) and have less effect as the animals grow older. In animals greater than 90 days, the pretraining experience has a diminishing effect on variability as age increases. This age-schedule interaction is being studied at present in an attempt to define the schedule-variability relationship.

Chronic extinction of behavior maintained by food and intracranial stimulation was affected by the injection of atropine. Doses of 4 mg/kg and 10 mg/kg were given to rats performing on an FR-10-chronic extinction chain for intracranial self-stimulation and for food pellet reward. Four mg/kg failed to affect performance maintained by ICS but caused post-reinforcement pausing food reward as well as prolonging extinction. Ten mg/kg increased the response rate for ICS and prolonged responses to extinction, while it slowed rate drastically for food by inducing very long postreinforcement pausing. Responses to extinction on pellet-maintained behavior was increased as much as ten-fold.

An experimental chamber which measures general activity has been designed and tested for its reliability. This chamber is in the process of being used to measure the effects of various drugs upon the general activity of prenatally irradiated and normal rats.

Numerous reports in the literature describe the effects of various drugs upon the general activity of normal rats. However, there is a paucity of information on the activity effect of drugs in a prenatally irradiated rat. The present study is an attempt to record the effect of several drugs from different drug classes upon the general activity of prenatally irradiated and normal rats. An experimental chamber has been developed to measure general activity by placing photoelectric cells at various points along the chamber. Activity is counting the number of times the light sources are interrupted. After receiving either 50, 100, or 150 r x-irradiation on the 16th day of prenatal development, the animals were maintained continuously in the activity box with food and water always available. Once a stable record had been obtained, the subjects received an intraperitoneal injection of a given drug. The following drugs and dosages were used:

<u>drug</u>	<u>dose (mg/kg)</u>
d-amphetamine	1, 3, and 6
seryanol	2, 10, and 20
meprobamate	50, 120, and 200
phenobarbital	10, 40, and 50

Present results indicate consistent and reliable measures of activity in the newly designed chamber with normal rats tested during conditions of food deprivation. Before deprivation hourly counts averaged 60-65 while hourly counts during deprivation days averaged 75-80. Administration of 3 mg/kg of d-amphetamine showed counts of over 600/hour for at least 2 hours after drug administration. An injection of saline produced an average count of 82 compared to a baseline count of 65. The remaining experimental conditions are in progress.

Two procedures have been developed for studying the relearning of new behavioral repertoires. With the first procedure, monkeys were required to learn repeatedly a new behavioral chain. The animals were trained in a chamber containing four groups of three levers. During each daily session the monkey's task was to learn a new four response chain by pressing in sequence the correct lever from each group. After a stable pattern of relearning was established, the number of incorrect responses declined to a steady state. Then this steady but changeable state was used as the dependent variable to study such factors as instruction stimuli, duration of time out for errors, forward versus backward sequences of learning, and superstitious chaining. It was found that the instruction stimuli did not improve the rate of learning. The monkeys either followed the instruction stimuli exactly and did not learn the sequence of responses, or they failed to attend to the stimuli and therefore learned at the same rate as when the stimuli were absent. The effect of a time out for errors was substantially greater. The time out procedure arranged that pressing an incorrect lever produced a stimulus which eliminated any possibility for reinforcement for a fixed time period. The effect of this basic procedure was to reduce sharply the number of incorrect responses. However, the duration of the time out was non-critical; in the range from 1 second to 240 seconds, all durations were equally effective in reducing error. The function of the time out was to reduce the amount of superstitious chaining. An experimental comparison of forward versus backward learning of the chain produced an unexpected result. It is generally believed that learning a chain backward (i.e., learning the final member first) is a superior approach. However, the results showed that the forward and the backward sequences did not produce significantly different numbers of incorrect responses. In a final experiment, still in progress, the effect of a large behavioral work requirement was investigated. It had been observed previously that

a major impediment to an error-free performance was the development of superstitious chains (i.e., the monkey made many extra responses which were not actually part of the chain but which intruded consistently as part of the performance.) The experiment was based on the theory that a large work requirement should reduce the probability of the superstitious chains. When the number of responses in each member of the chain was varied from 1 to 20, it was indeed observed that the number of errors due to superstitious chaining was reduced by the large work requirement.

The second basic procedure concerned with relearning involved the repeated acquisition of new stimulus discriminations. Monkeys were trained to press a center lever in order to produce and to change stimuli, such as a triangle, a square, a circle, etc. On a given session, one of these stimuli sets the occasion for reinforcement; that is, when this stimulus is present, a response on a second lever produces a food reinforcement. Since the correct stimulus is different for every session, the monkey is required to develop a flexible, learning-how-to-learn repertoire which is similar in basic principle to the experiment described above on the relearning of behavioral chains. In the stimulus discrimination experiment, the basic procedure has been established along with several important refinements, such as the response requirements, the sequence of stimuli, and the environmental consequences of responding to incorrect stimuli. An experiment now in progress is investigating the effects of the work requirement contingent upon a response to an incorrect stimulus.

The adjusting avoidance procedure, a technique previously used for inducing stress and fatigue, has been investigated further. With this procedure each avoidance response accumulates a constant amount of shock-free time (the time base) and stimuli indicate the amount of shock-free time remaining. When the time base was increased parametrically from 2 to 20 seconds, the avoidance response rate and the shock frequency decreased systematically. In contrast, the point at which the subject began to respond (in terms of temporal steps from the shock) was essentially unchanged. This finding indicates that the stimulus discrimination underlying the basic reaction to the adjusting avoidance situation is probably prepotent and that this discrimination was effective enough to negate any changes in the time base.

Rhesus monkeys have been trained on a procedure which requires the animal to perform a "matching-to-sample" problem to obtain food and water reinforcements. This problem is placed on an "adjusting difficulty" program, depending upon the animal's performance. In the next phase of the experiment avoidance requirements will be superimposed on this behavior to provide information about deterioration of the matching-to-sample behavior during prolonged stress.

A colony of two male and one female chimpanzees has been established to study the effects of confinement and limited "social" contact on a baseline performance requirement. Each animal is assigned his or her own work quarters to obtain their basic diet by a DRL procedure. This performance will then be studied as a function of specific requirements on the DRL performance itself and other variables such as activities of their colony mates.

A related social experiment is investigating the conditions under which two monkeys will reinforce each other (i.e., one monkey presses a lever and thereby gives the other monkey a food pellet). Conditions sufficient to establish and maintain the cross-reinforcement relation have been found. Individual pretraining on the basic task, alteration of reinforcements, close proximity of the two monkeys, and an immediate conditioned reinforcement for the responding monkey seem to provide sufficient conditions. Experiments now in progress are aimed at separating the necessary from the sufficient conditions.

The experiments described above were aimed at the study and analysis of complex and basic behavioral repertoires. Some of these repertoires have been applied to the behavioral analysis of stress and fatigue. The next section of this report will deal with methodology for producing stress and fatigue and the behavioral consequences as determined by disruption of complex behavioral repertoires.

Prolonged sessions during which the animal subject must work continuously to avoid an electric shock has provided the basic stress-inducing method in this laboratory. As a substantial extension of this procedure, monkeys were exposed to cycles of three days of the avoidance procedure followed by four days of rest. The cycle was repeated for more than a year in order to assess the long-term effects of chronic stress. During the first ten cycles, the avoidance response rate increased and the shock frequency declined sharply. Then the behavior reached a final stable state which was maintained for the remainder of the experimental year. Although the expected increase in 17-OH-corticosteroids due to the avoidance stress occurred during the first ten cycles, the hormonal response decreased with repeated cycles until finally the avoidance session had no effect on steroid output. The effect of the chronic avoidance stress apparently adapted out as a function of prolonged and frequent repetition.

A paced avoidance procedure has been used as a method both for producing stress and for measuring the effects. The procedure required that the monkey either press his lever during a narrow time interval or receive a shock. The trained monkey reacted to this procedure by timing his responses so that they fell within the narrow safe periods. When a continuous five day session was run, the timing behavior held up well for the first three days. By the fourth and fifth days, however, a general increase in shock frequency and the

variability of the timing behavior was observed. This finding was interpreted as a fatigue-induced decrement in the timing performance. The performance recovered completely after two days of rest.

A "vigilance" method to produce stress and fatigue and to yield a new behavioral measure of the effects has been developed. The procedure requires the monkey to press a lever only during the presentation of a dim light which is turned on at unpredictable times during the session. If the monkey responds within 20 seconds after the light goes on, he will not be shocked. In order to increase the sensitivity and the stress inducing aspects of the procedure, the light intensity is adjusted automatically to be quite near the animals absolute threshold. If the monkey performs well, the light intensity is reduced; if he performs poorly, the intensity is raised. Thus, it is possible to coerce the monkey to work continuously near the limits of his performance.

The effects of another form of stress, conditioned fear, were studied in an experiment with rats. The animals were trained on a two-response timing procedure which required that response B follow response A by more than a certain delay. When a 5 minute clicker was followed by an electric shock (the condition fear procedure) the frequency of A to B response sequences was reduced sharply. However, the accuracy of the timing behavior, when it occurred, was unchanged.

Summary and Conclusions: The progress reported above may be summarized under three aspects of the program.

- 1) The establishment and study of complex behavioral repertoires, including behaviors of basic scientific interest. The behaviors investigated in this report period include timing behavior, schedule preference, performance and retention during hibernation, stimulus generalization, behavioral variability, chronic extinction, general activity, acquisition of new behavioral chains and new discriminations, adjusting avoidance, matching-to-sample, and basic social behavior among primates.
- 2) The investigation of methodology for producing stress and fatigue. Under this category, experiments have been done on extended cycles of 3-day avoidance sessions, paced avoidance during 5-day sessions, vigilance with required observation of a near threshold light, and a conditioned fear technique.
- 3) The analysis of the effects of stress and fatigue in terms of decrements in the performance of behavioral repertoires. Studies in this area have focused primarily upon the disruption of timing behavior.

ARMY RESEARCH TASK REPORT			REPORTS CONTROL SYMBOL CSCRD-6(R2)	
ACCESSION NUMBER <div style="text-align: right;">36175</div>		PROJECT, TASK, OR SUB TASK NO. <div style="text-align: right;">3A012501B8130803</div>		
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D. C., 20315		2. FUNDING AGENCY Army Medical R&D Command Office of The Surgeon General Washington, D. C., 20315		
3. CONTRACTING AGENCY NA		4. CONTRACTOR AND/OR GOV'T LABORATORY A Walter Reed Army Inst of Rsch Walter Reed Army Medical Center Washington, D. C., 20012 723-1000, Ext 3552		
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Mason, John W., M.D., Dept of Neuroendocrinology Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012 576-3559 or Interdepartmental Code 198, Ext 3559 See Continuation Sheet				
6. TITLE OF: PROJECT <input type="checkbox"/> <div style="margin-left: 40px;"> TASK <input type="checkbox"/> Analysis of behavior and of mediating mechanisms: SUBTASK <input checked="" type="checkbox"/> neuroendocrinological factors (U) </div>				
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964				
8. RESUME' (U) This program continues to be concerned with the neuroendocrine integration of physiological and biochemical responses to stress. Much additional data has been obtained within the past year supporting and developing further our major working hypothesis that the study of the overall balance between the many interrelated hormones may provide unique insight into the operation of mechanisms integrating and coordinating bodily functions. Studies involving concurrent measurement of about twelve different hormones during psychological stress and physical stresses such as hemorrhage, muscular exertion, cold, and trauma have shown that stereotyped hormone response patterns occur in each of these situations. The concept suggested is that since every hormone changes, and since hormones have wide spectrums of overlapping metabolic and physiological effects, then the bodily changes in stress must be regarded as the <u>net</u> result of the overall changes in balance between <u>all</u> the hormones. These patterns differ somewhat from one situation to the next apparently in accordance with the specific metabolic needs which are imposed. It is especially noteworthy that every hormone studied in each situation fluctuates significantly both during and following the stress. This work is generating some new concepts of bodily organization and a monograph summarizing the present status of this approach is now in preparation.				
9. KEY WORDS Stress, emotion, psychosomatic, neuroendocrine, hormones, integration.				
10. SUPPORTING PROJECTS Not applicable				
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet		12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet		
<div style="display: flex; justify-content: space-between;"> <div>DA FORM 1309R 1 June 63</div> <div>PREVIOUS EDITIONS ARE OBSOLETE</div> <div>PAGE 1 of ____</div> </div>				

ACCESSION NO. 36175		ARMY RESEARCH TASK REPORT	
13. PROJECT, TASK OR SUBTASK NUMBER	11 22 23 24 25 26 27 28 29 3 A 0 1 2 5 0 1 B 8 1 3 0 8 0 3		
14. DATE OF REPORT (30-33)	30 33 34 0 6 6 4 4		
15. SECURITY OF WORK (34)			
16. TYPE OF REPORT	35 36 47 48 49 50 51 52 55 3 1 2 6 3		
17. SCIENTIFIC FIELD a. Topical Classific. (56-61) b. Functional Class (62-64)	56 61 62 64 0 1 0 6 0 5		
18. OSD CLASSIFICATION (65-66)	65 66 67 B R 1		
19. R&D CATEGORY (67)			
20. CONTRACT NUMBER	11 12 13 14 15 17 18 21 22 26 27 D A		
21. GRANT NUMBER	28 29 30 33 34 35 36 38 39 40 41 45 46 D A G		
22. ESTIMATED COMPLET. DATES	47 51 52 56 57 61 62 66 67 71 1 C O N T 2 3 4 5		
23. PRIORITY (11-14)	11 14 15 26 2 6 • 1 1 • 2 5 • 0 1 • 1		
24. PROGRAM ELEMENT (15-26)			
25. CMR&D CODES	27 29 30 32 33 35 N / A		
26. CDOG REFERENCE a. Paragraph No. (36-44) b. Functional Group (45)	36 39 40 41 42 43 44 45 1 4 1 2 a 6		
27. FUNDING a. Est. Total Cost (11-15) b. % Spent Intern. (16-18) " " Extern. (19-21) c. Total Obligation (22-26) d. Progmd. Cur. FY (27-33) e. " " " +1 (34-40) f. " " " +2 (41-47) g. " " " +3 (48-54) h. " " " +4 (55-61) i. " " " +5 (62-68) j. " " " +6 (69-75) k. Total Man Years of Effort (76-78)	11 15 16 18 19 21 22 26 1 2 27 28 29 33 34 35 36 40 41 42 43 47 48 49 50 54 55 56 57 61 62 63 64 68 69 70 71 75 76 78		

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

- (P) Brady, J. V., Lt Col, MSC, Dept of Experimental Psychology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-2483 or Interdepartmental Code 198, Ext 2483 49
- (A) Werdegarr, D., Capt, MC, Dept of Neuroendocrinology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-3559 or Interdepartmental Code 198, Ext 3559 49
(Presently with Dept of Medicine, University of California
Medical Center, San Francisco, Calif.)
- (A) Miller, R. E., M.D., Dept of Neuroendocrinology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-3029 or Interdepartmental Code 198, Ext 3029 49
- (A) McHugh, P. R., M.D., Dept of Neuroendocrinology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-3027 or Interdepartmental Code 198, Ext 3027 49
(On grant from Howard University, Washington, D. C.)
- (A) Smith, G., Capt, MC, Dept of Neuroendocrinology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-3027 or Interdepartmental Code 198, Ext 3027 49
- (A) Tolson, W. W., PhD., Dept of Neuroendocrinology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-3559 or Interdepartmental Code 198, Ext 3559 49
(Presently with Food and Drug Administration,
Washington, D. C.)
- (A) Mougey, E. H., M.S., Dept of Neuroendocrinology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-3025 or Interdepartmental Code 198, Ext 3025 49
- (A) Taylor, E. D., M.S., Dept of Neuroendocrinology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-3026 or Interdepartmental Code 198, Ext 3026 49
- (A) Wherry, F. E., B.S., Dept of Neuroendocrinology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-3029 or Interdepartmental Code 198, Ext 3029 49
- (A) Jones, J. A., M.A., Dept of Neuroendocrinology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-3005 or Interdepartmental Code 198, Ext 3005 49
(On grant from Washington School of Psychiatry,
Washington, D. C.)
- (A) Johnson, T. R., B.S., Dept of Neuroendocrinology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-3025 or Interdepartmental Code 198, Ext 3025 49

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

(A) Robinson, J. A., B.S., Dept of Neurology and Psychiatry
Howard University, College of Medicine, Washington, D.C., 20001
797-1636 or Interdepartmental Code 151, Ext 636 49
(On contract with Howard University)

(A) Gilmore, L. I., Dept of Neuroendocrinology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-3026 or Interdepartmental Code 198, Ext 3026 49
(Working at Howard University, Washington, D. C.)

(A) Black, W. C., Capt, MSC, Dept of Experimental Psychology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-3031 or Interdepartmental Code 151, Ext 3031 49

(A) Beer, B., M.A., Dept of Experimental Psychology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-3031 or Interdepartmental Code 198, Ext 3031 49

REPORTS. Annual Progress Report, Walter Reed Army Institute of
Research, 1 July 1963 - 30 June 1964.

Bunney, W. E. Jr., Mason, J. W. and Hamburg, D. A.: Correlations
Between Behavioral Variables and Urinary 17-Hydroxycorticosteroids
in Depressed Patients. Psychosom. Med., 25:488, 1963. (Abstract)

Friedman, S. B., Mason, J. W. and Hamburg, D. A.: Urinary 17-
Hydroxycorticosteroid Levels in Parents of Children with Neoplastic
Disease. Psychosom. Med., 25:364-376, 1963.

Friedman, S. B., Chodoff, P., Mason, J. W. and Hamburg, D. A.:
Behavioral Observations in Parents Anticipating the Death of a
Child. Pediatrics, 32:610-625, 1963.

McHugh, P. R., Black, W. C. and Mason, J. W.: Some Hormonal
Effects of Electrical "Self Stimulation" in the Lateral Preoptic
Region. The Physiologist, 6:232, 1963. (Abstract)

Mason, J. W.: Comments on the Psychoendocrine Approach to Psycho-
somatic Research (Discussion). Proceedings of the Forty-fourth
Ross Conference on Pediatric Research, Columbus, Ohio., 33-37, 1963.

Mougey, E. H. and Mason, J. W.: Separation of Some Iodoamino Acids
and Iodide by Gel Filtration. Anal. Biochem., 6:223-233, 1963.

Penick, S. B., Smith, G. P., Wieneke, K. and Himble, L. E. Jr.:
An Experimental Evaluation of the Relationship Between Hunger and
Gastric Motility. Am. J. Physiol., 205:421-426, 1963.

Sachar, E. J., Mason, J. W., Kolmer, H. S. Jr. and Artis, K. L.:
Psychoendocrine Aspects of Acute Schizophrenic Reactions.
Psychosom. Med., 25:510-537, 1963.

Smith, G. P. and Mason, J. W.: The Acidity of Gastric Contents of *Macaca Mulatta*. *The Physiologist*, 6:277, 1963. (Abstract)

Wadeson, R. W., Mason, J. W., Hamburg, D. A. and Handlon, J. H.: Plasma and Urinary 17-OH-CS Responses to Motion Pictures. *Arch. Gen. Psychiat.*, 9:146-156, 1963.

Wolff, C. T., Mason, J. W., Friedman, S. B. and Hofer, M. A.: The Relationship Between Ego Defenses and the Adrenal Response to the Prolonged Threat of Loss: A Predictive Study. *Psychosom. Med.*, 25:497, 1963. (Abstract)

ANNUAL PROGRESS REPORT

Project No. 3A012501B813	Title: Army Medical Basic Research in Life Sciences
Task No. 08	Title: Neuropsychiatry
Subtask No. 03	Title: Analysis of behavior and of mediating mechanisms: Neuroendocrinological factors ()

Description:

In experiments with both monkey and human subjects, patterns of hormonal change have been observed during psychological and physical stress. Psychological stress experiments have involved hormonal balance studies in monkeys during acute and chronic conditioned emotional disturbances and in humans such as the parents of leukemic children, medical, neurological and surgical patients, and normal subjects exposed to laboratory social stress situations. Physical stress experiments have involved hormonal balance studies in the monkey during hemorrhage, muscular work, food deprivation, and cold, as well as studies of surgical trauma in the human. Work on the development or refinement of hormone assay procedures and basic neuroendocrine physiological studies necessary for the interpretation of stress experiments have continued.

Progress:

Hormonal Balance in Psychological Stress

A large study involving measurement of 17-hydroxycorticosteroids (17-OH-CS), epinephrine, norepinephrine, thyroid hormones, estrogens, androgens, and aldosterone during 72-hour conditioned avoidance sessions in the monkey have been completed. An analysis of the direction and timing of each hormonal change during and following avoidance, taken together with knowledge of the metabolic effects of each hormone, has led to a tentative interpretation of the hormonal pattern as being oriented generally in terms of catabolic energy production and water retention during the conditioned emotional disturbance, with a long recovery period in which anabolic or restorative processes are predominant. The addition of insulin measurements to these experiments during the past year lends strong further support to this interpretation, since insulin levels appear to drop during avoidance but then rise strikingly above normal in the aftermath, in some instances for longer than ten days. We hope soon to provide another predictive test of the hypothesis when a new radio-immunochemical assay for growth hormone, now under development by Mrs. Wherry, becomes available. A pattern of hormonal change in monkeys adapting to the restraining chair which appears extremely similar to the avoidance pattern has been observed in a large series of experiments, lending further support to relating this pattern primarily to psychological factors.

It has been observed that the normal phasic pattern of hormonal change to acute stress lends itself to marked derangement when stress is scheduled on a chronic or repetitive basis. Such disturbances of normal hormonal balance may vary from sustained or progressively increasing changes, when avoidance stress is programmed on a six-hour on--six-hour off basis, to paradoxical responses, decreases rather than increases in particular hormones, when three-day avoidance and four-day rest periods are continuously alternated, for example. A major future goal is the study of whether such hormone imbalances, psychologically induced, eventually are associated with somatic illness or disease. Such studies will require considerable space and time and have remained limited during the past year because of continued delays in building alterations. One monkey has been observed, however, in which a hormonal response pattern including sustained 17-OH-CS, epinephrine, and vasopressin elevations, with a progressive rise in aldosterone and norepinephrine, and a drop in estrogens, was associated with progressive dependent edema beginning shortly after a chronic avoidance program was initiated and disappearing within a week after avoidance was terminated. Similar observations in animals developing infections, peptic ulcer, cardiovascular, or other diseases are hopefully forthcoming.

Studies of hormonal patterns in medical patients will provide an ultimate test for the psychoendocrine approach in the psychosomatic field. Lacking local clinical facilities, a collaborative project has been initiated during the past year with the Medical and Psychiatric Services at the Howard Medical School in which overall hormonal balance is being studied in a variety of medical patients including those with hypertension, peptic ulcer, obesity, rheumatoid arthritis, atopic dermatitis, and several other diagnostic categories. Bizarre disturbances in basal hormonal balance have already been observed in these patients, as well as highly abnormal hormonal response patterns to acute psychological stress. For example, it was observed that an obese woman maintained a chronic basal hormone pattern in which androgen excretion was about five times normal while 17-OH-CS, catecholamine and estrogen levels were close to the normal range. During a period of acute psychological upset, however, while the 17-OH-CS, epinephrine and norepinephrine rose as usually seen in normals, androgen levels showed a marked increase instead of the normal decrease, and estradiol and estrone were dissociated in an extraordinary way. Psychoendocrine study is then aimed at both chronic basal hormone balance and hormone response pattern to psychological distress. These studies clearly appear to provide a new and promising approach to the investigation of psychosomatic processes and may eventually yield diagnostic and possibly therapeutic capabilities in the psychosomatic field which are badly needed.

Clinical psychoendocrine projects aimed primarily at using adrenal hormone measurements as indices of emotional state in the study of both

situational and personality factors relating to emotion include newly initiated experiments with epileptic patients during psychotherapy, studies of violent and aggressive behavior in adolescent boys, studies of normal young adults interacting socially in a laboratory yoked-avoidance situation, as well as follow-up studies on parents of leukemic children. An analysis of MMPI (Minnesota-Multiphasic Personality Inventory) results on 20 of these parents has shown recently that a highly significant correlation exists between the K-score and chronic 17-OH-CS levels, indicating that subjects who characteristically have difficulty or reluctance in expressing emotional feelings or in ventilating grievances usually have high chronic mean 17-OH-CS levels. This measurement provides useful support for what was previously only a clinical impression. Follow-up studies indicate not only that parents who had high mean 17-OH-CS levels during their child's illness show declining levels following the child's death, but that many subjects having low 17-OH-CS levels prior to the child's death show rising levels in the follow-up period. Analysis of correlating psychiatric data is currently under way.

Hormonal Balance in Physical Stress

Hemorrhage. Analysis of hormonal response pattern so far confirms earlier work reporting elevations in 17-OH-CS, aldosterone, and catecholamine levels in hemorrhage, and in addition shows an acute brief increase followed by a decline in plasma insulin level and a 50% drop in thyroid hormone levels. So far then, this pattern differs in several ways from that observed in psychological stress--aldosterone, thyroxine, and insulin responding initially in the opposite direction from that observed in psychological stress. The pattern suggests that these specific changes may be related to the specific metabolic needs centering around oxygen insufficiency and fluid loss. Further experiments are planned in which these metabolic disturbances will be studied separately in an effort to evaluate more conclusively the interpretation of the pattern data.

Muscular Work. A series of experiments on the effects of weight-lifting exertion on hormone levels in the monkey have been completed. Analysis of the 17-OH-CS data indicates physical exercise per se may exert a relatively small influence on urinary 17-OH-CS excretion, but that a major problem in the neuroendocrine study of physical stress in the conscious animal is to separate the psychological from the physical aspects of the experimental situation. In several animals, the highest 17-OH-CS elevations were observed on the days when least work was performed, presumably related to an emotional response in association with refusal of the animal to lift heavy weights in order to obtain food. In general, the hormone response pattern in this exercise situation is similar to that observed in psychological stress, but this conclusion is considered quite tentative until the psychological component can be better evaluated in related experiments. Two other approaches are

being tested. First, monkeys climbing 1000-2000 meters per day for food reward appear to show less evidence of psychological upset than the weight-lifting monkeys and are involved in a more natural form of activity. Since 17-OH-CS responses are relatively mild in those animals, hormone patterns are now being determined in selected experiments. Secondly, hormone response patterns are being studied during treadmill exercise in human subjects, so that it will be possible to make a more extensive evaluation of psychological factors.

Trauma. Hormonal patterns have been studied in six patients undergoing surgical repair of ruptured intervertebral discs. Curiously, a number of patients screened during this study showed minimal, if any, 17-OH-CS elevations in spite of the considerable tissue trauma inflicted, raising again the question of psychological factors as a component of the surgical stress situation. Those five subjects selected as having the largest 17-OH-CS responses showed a hormonal pattern very similar to that seen in psychological stress, except for a failure of androgens to decrease in some cases. The patterns of one patient showing minimal 17-OH-CS changes is now also being studied to determine whether other hormonal changes related to tissue damage or repair might be detected. Continuation of these studies will be in the direction of separating out the multiple components of the surgical stress situation, such as tissue damage, hemorrhage, fluid imbalance, immobilization, pain and psychological reactions, etc., and attempting to deal with simpler stimulus variables. Several of these variables have already been under analysis as indicated in related studies.

Cold. After considerable difficulty in devising suitable experimental conditions, samples have been recently obtained on a monkey exposed to a temperature of 10°C for three days. While a moderate gradual increase in 17-OH-CS levels occurred, very little increase in thyroid hormone levels was observed, possibly because the temperature drop was excessive for the monkey. The remainder of the hormone pattern is being determined and similar experiments are planned with less marked temperature drops.

Others. It appears increasingly that in order to test the general validity of the "overall hormone balance" concept, it will be necessary to study hormone response patterns not only in natural physical stress situations, but to selected metabolic stimuli as well. An extensive literature review now under way of the metabolic effects of hormones and of interactions, synergetic and antagonistic, between hormones is providing a solid basis for the planning and design of crucial experiments to test this developing concept of bodily integration.

Hormonal Patterns and Neural Mechanisms

Brain Stimulation. The study of hormonal patterns in five monkeys during prolonged periods of self-administered electrical stimulation in

the medial forebrain bundle has been completed. A pattern of change very similar to that observed in psychological stress was found, with elevations in 17-OH-CS, epinephrine, norepinephrine and thyroid hormone levels and a decrease in estrogen levels. It is of great interest that this entire pattern can be reproduced by the stimulation of a single brain area, suggesting perhaps that the mediating neural apparatus elicits the rather complex but stereotyped response pattern as a unit.

Studies on the influence of the amygdaloid complex on pituitary-adrenal cortical function have continued, with particular attention to the site of humoral feed-back effects of cortisol on the central nervous system. It has been shown that sudden injections of cortisol may substantially diminish the 17-OH-CS response to amygdaloid, but not hypothalamic, stimulation. The latter effect indicates that neural influences may take precedence over humoral regulatory factors. Simultaneous electrical recording from amygdala and hypothalamus indicates that stimulation parameters must be such as to induce after-discharges in the amygdala, usually reflected also in the hypothalamus, in order for ACTH release to occur. Electrical stimulation of the amygdala has also been shown to inhibit gastric secretory activity at the same time 17-OH-CS levels rise, in related experiments.

Brain Lesions. The study of nine experiments in four bilaterally amygdalectomized monkeys indicates that such lesions dramatically alter hormonal response patterns to 72-hour avoidance sessions. Virtually every hormone is affected to some degree, but especially striking have been the exaggerated androgen and insulin responses during the post-avoidance recovery period. Androgen levels may rise to about three times normal levels and the rise may begin on the first recovery day rather than during the three to six day period as in normals. While 17-OH-CS levels show a diminished response the first day of avoidance, there is subsequently on the second and third days a delayed and prolonged rise greater than normal. One possible explanation for this puzzling finding is that it relates to the absence of amygdaloid input to the hippocampus and that the normal delayed suppression or feed-back function of the latter structure is impaired. This possibility is considered worthy of further study by means of fornix section, alone and in combination with amygdalectomy, and by electrical recording from the hippocampus in normal and amygdalectomized animals during avoidance.

Both the brain stimulation and ablation work continue to build support for the concept of an important functional role of the amygdala and the hippocampus, acting together in a coordinated fashion to provide both an initial facilitory and a secondary and prolonged inhibitory influence on hormone secretions. It will require further experiments to determine the full extent to which this mechanism is involved in the integration of the hormonal response pattern observed in psychological stress, but our present evidence indicates that the limbic system role is a substantial one.

Biochemical Methodology. There is continued heavy emphasis on the development and improvement of hormone assay procedures since it appears increasingly that a major factor limiting widespread application of the psychoendocrine approach in psychiatry and medicine is the exceptionally difficult, costly and time-consuming nature of most present hormone assay procedures. During the past year a modification of the Yalow-Berson radio-immunochemical method for plasma insulin measurement has been successfully set up by Mrs. Wherry and validated in an exhaustive series of chemical and physiological studies. This method permits the precise measurement of less than .001 micrograms of insulin. At present a similar technique for measurement of growth hormone is being evaluated, with encouraging progress being made. With proper support, it appears very likely that this new analytical principle can be applied to the measurement of many other peptide hormones, including glucagon, parathyroid hormone, the gonadotrophins, ACTH, TSH, and others. A new method for the separate measurement of thyroxine and triiodothyronine has been developed by Mr. Mougey and has been fully validated in chemical and physiological studies. A new method involving dialysis and isotope techniques for the measurement of plasma 17-OH-CS levels has been checked and found to give closely similar results to the Nelson-Samuels procedure (correlation coefficient $r=.91$). It permits the assay of about five times as many samples as the older method and requires only about one-tenth as much plasma.

Physiological Studies. A large series of experiments on the diurnal variation of hormone balance in normal animals, an essential body of normative data for the interpretation of stress experiments, has been completed. All hormones studied, with the possible exception of thyroxine, have a marked and consistent diurnal rhythm with characteristic peaks at various times in the diurnal cycle. Estradiol, for example, appears to peak during the late evening while epinephrine and norepinephrine peak in the early and late morning, respectively. All acute experiments must be done against this background of diurnal change.

Injectons of T3 appear to markedly suppress T4 levels and we have obtained evidence that this effect may be directly on the thyroid and not through the pituitary and the TSH mechanism. T3 output, therefore, must always be considered when the BEI or thyroxine levels are shown to decrease as, for example, in hemorrhage.

Injectons of glucose produce marked increases in plasma insulin levels in the monkey, with the peak usually occurring in about 30 minutes. Such studies provide a basis for estimating dynamics of insulin secretion in the design of sample collection for various stress experiments. Our conventional two-hour sample for adrenal cortical hormones, for example, is not suitable for insulin regulation studies.

In order to evaluate the role of purely humoral mechanisms in hormonal response patterns to stress, a series of infusion experiments has been initiated in which the effects upon the overall hormone pattern resulting from infusion of a single hormone, such as cortisol or epinephrine, are being studied. Such experiments may be of particular value in separating out the phasic changes in hormone balance during stress, in terms of those changes which result from humoral or metabolic changes secondary to the initial hormonal responses from those effects which are related directly to primary central integrating mechanisms. It will be of considerable interest, for example, to determine if the anabolic rebound of androgen and insulin occur simply following the catabolic action of epinephrine or cortisol.

Summary and Conclusions:

This program continues to be concerned with the neuroendocrine integration of physiological and biochemical responses to stress. A large additional body of data has been obtained within the past year supporting and developing further our major working hypothesis that the study of the overall balance between the many interrelated hormones may provide unique insight into the operation of mechanisms integrating and coordinating bodily functions.

Studies involving concurrent measurement of about twelve different hormones during psychological stress and physical stresses such as hemorrhage, muscular exertion, cold, and trauma have shown that stereotyped hormone response patterns occur in each of these situations. The concept suggested is that since every hormone changes, and since each hormone has a wide spectrum of metabolic and physiological effects, then the bodily changes in stress must be regarded as the net result of the overall changes in balance between all the hormones. These patterns differ somewhat from one situation to the next apparently in accordance with the specific metabolic needs which are imposed. It is especially noteworthy that every hormone studied in each situation fluctuates significantly in one direction or another, both during and for a surprisingly long time following the stress. It is believed that this work is generating some rather new and basic concepts of bodily organization and a monograph summarizing the present status of this approach is now in preparation.

ARMY RESEARCH TASK REPORT				REPORTS CONTROL SYMBOL CSCRD-6(R2)	
ACCESSION NUMBER 36176			PROJECT, TASK, OR SUBTASK NO. 3A012501B8130804		
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D. C., 20315			2. FUNDING AGENCY Army Medical R&D Command Office of The Surgeon General Washington, D. C., 20315		
3. CONTRACTING AGENCY NA			4. CONTRACTOR AND/OR GOV'T LABORATORY A Walter Reed Army Inst of Rsch Walter Reed Army Medical Center Washington, D. C., 20012 723-1000, Ext 3552		
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Armington, J. C., Ph.D., Dept of Sensory Psychology Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012 576-5260 or Interdepartmental Code 198, Ext 5260 See Continuation Sheet 49					
6. TITLE OF: PROJECT <input type="checkbox"/> TASK <input type="checkbox"/> Analysis of behavior and of mediating mechanisms: SUBTASK <input checked="" type="checkbox"/> Psychophysical and electrophysical data correlation (U)					
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964					
8. RESUME' (U) The present report is concerned with the relation of electro-physiological measures to behavior with particular emphasis on the visual system. The registration of a stimulus, its modification by receptor structure, the particular neural coding along the afferent pathway together with its ultimate production in measurable behavior are especially emphasized. To this end a variety of techniques and animals are used, each affording an advantage for the problem under investigation.					
9. KEY WORDS Psychology, sensory, electrophysical, visual, eye, ophthalmic, optic, night blindness, color blindness.					
10. SUPPORTING PROJECTS Not Applicable					
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet			12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet		
<div style="display: flex; justify-content: space-between;"> FORM 1309R 1 June 63 PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of ____ </div>					

ACCESSION NO. 36176		ARMY RESEARCH TASK REPORT	
13. PROJECT, TASK OR SUBTASK NUMBER		11 22 23 24 25 26 27 28 29 3 A 0 1 2 5 0 1 B 8 1 3 0 8 0 4	
14. DATE OF REPORT (30-33)		30 33 34	
15. SECURITY OF WORK (34)		0 6 6 4 4	
16. TYPE OF REPORT		35 36 47 48 49 50 51 52 53 3	
17. SCIENTIFIC FIELD a. Topical Classification (34-61) b. Functional Class (62-64)		56 61 62 64 0 1 0 6 0 5	
18. OSD CLASSIFICATION (65-66)		65 66 67	
19. R&D CATEGORY (67)		B R 1	
20. CONTRACT NUMBER		11 12 13 14 15 17 18 21 22 26 27 D A	
21. GRANT NUMBER		28 29 30 33 34 35 36 38 39 40 41 45 46 D A G	
22. ESTIMATED COMPLET. DATES		47 51 52 56 57 61 62 66 67 71 1 C O N T 2 3 4 5	
23. PRIORITY (11-14)		11 14 15 26	
24. PROGRAM ELEMENT (15-26)		6 1 1 2 5 0 1 1	
25. CMR&D CODES		27 29 30 32 33 35 N / A	
26. CDOG REFERENCE a. Paragraph No. (36-44) b. Functional Group (45)		36 39 40 41 42 43 44 45 1 4 1 2 a 6	
27. FUNDING a. Est. Total Cost (11-15) b. % Spent Intern. (16-18) " Extern. (19-21) c. Total Obligation (22-26) d. Programd. Cur. FY (27-33) e. " " " +1 (34-40) f. " " " +2 (41-47) g. " " " +3 (48-54) h. " " " +4 (55-61) i. " " " +5 (62-68) j. " " " +6 (69-75) k. Total Man Years of Effort (76-78)		11 15 16 18 19 21 22 26 1 2 27 28 29 33 34 35 36 40 41 42 43 47 48 49 50 54 55 56 57 61 62 63 64 68 69 70 71 75 76 78	

DA FORM 1309R
1 June 63

Previous Editions are Obsolete

Page 2 of _____

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

- (P) Biersdorf, W. R., Ph.D., Dept of Sensory Psychology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-5260 or Interdepartmental Code 198, Ext 5260 49
- (P) Granda, A.M., Ph.D., Dept of Sensory Psychology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-5260 or Interdepartmental Code 198, Ext 5260 49
- (P) Barnet, A. B., M.D., Dept of Sensory Psychology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D.C., 20012
576-5260 or Interdepartmental Code 198, Ext 5260 49
(Washington School of Psychiatry, 1610 New Hampshire
Ave., Washington, D. C., 20009)
- (P) Chapman, R. M., Ph.D., Dept of Sensory Psychology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-5260 or Interdepartmental Code 198, Ext 5260 49
(Institute of Behavioral Research, 2426 Linden Lane,
Silver Spring, Maryland)
- (P) Krauskopf, J., Ph.D., Dept of Sensory Psychology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-5260 or Interdepartmental Code 198, Ext 5260 49
(Dept of Psychology, University of Maryland, College
Park, Maryland)
- (A) Lodge, A., Ph.D., Dept of Sensory Psychology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D.C., 20012
576-5260 or Interdepartmental Code 198, Ext 5260 49
(Washington School of Psychiatry, 1610 New Hampshire Ave.
Washington, D. C., 20009)
- (A) Matsumiya, Y., Ph.D., Dept of Sensory Psychology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-5260 or Interdepartmental Code 198, Ext 5260 49
(George Washington University, Washington, D. C.)
- (A) Graf, V., A.M., Dept of Sensory Psychology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-5260 or Interdepartmental Code 198, Ext 5260 49
(Dept of Psychology, University of Maryland, College
Park, Maryland)
- (A) Lall, A.E., A.M., Dept of Sensory Psychology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-5260 or Interdepartmental Code 198, Ext 5260 49
(Institute of Behavioral Research, 2426 Linden Lane,
Silver Spring, Maryland)
- (A) Goodwin, R., A.B., Dept of Sensory Psychology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-5260 or Interdepartmental Code 198, Ext 5260 49
(Washington School of Psychiatry, 1610 New Hampshire Ave.
Washington, D. C., 20009)

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

(A) Kropel, W. J., B.E.E., Dept of Sensory Psychology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-5260 or Interdepartmental Code 198, Ext 5260 49

(A) Bragdon, H., SP/7, Dept of Sensory Psychology
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-5260 or Interdepartmental Code 198, Ext 5260 49

REPORTS. Annual Progress Report, Walter Reed Army Institute of
Research, 1 July 1963 - 30 June 1964.

Armington, J. C., and Biersdorf, W. R.: Color Vision. Annual
Rev. Psychol., 14: 93-114, 1963.

Armington, J. C.: Relations between Electroretinograms and
Occipital Potentials Elicited by Flickering Stimuli. In Flicker
Proceedings of the Symposia on Physiology of Flicker and Flicker
Electroretinography, The Hague, Dr. W. Junk Publishers, 194-206,
1964.

Gouras, P., Armington, J.C., Kropfl, W., Tepas, D.I., and
Gunkel, R.D.: Evaluation of Visual Function in Man by Electronic
Computation of Evoked Potentials. Proceedings of the Conference
on Data Acquisition and Processing in Biology and Medicine.,
New York, Pergamon Press, 1963.

Granda, A.M. and Biersdorf, W.R.: Electrical Responses of the
Human Eye following Intense Chromatic Pre-exposures. Vision
Research, 3:431-445, 1963.

Granda, A.M. and Biersdorf, W.R.: Electrical Measurement of
Incremental Thresholds in the Human Eye. (Abstract). J. Opt. Soc.
Amer., 54:580, 1964.

Krauskopf, J.: Further Measurements of Human Retinal Images. J.
Opt. Soc. Amer., 54:715-716, 1964.

ANNUAL PROGRESS REPORT

Project No. 3A012501B813 Army Medical Basic Research in Life Sciences.

Task No. 08 Neuropsychiatry

Subtask No. 04 Analysis of behavior and mediating mechanisms: Psychophysical and electrophysiological data correlation

Description: The intent of this task is to correlate neurophysiological structure with categories of behavior and their assessment by means of electrical recording. Where applicable, behavioral problems are designed as corollary experiments. Emphasis is on sensory information processing, discrimination and learning, and central integrative networks.

Progress:

I. Studies of Peripheral Mechanisms

Human Visual System. 1) Measurement of light reflected from human fundus. A photoelectric ophthalmoscope developed to measure the light distribution in human retinal images has been used to measure the efficiency of different parts of the pupil. Two experimental arrangements were used. In one setup the light entered the pupil at different places in the pupil plane. In the other, the light was collected from different parts of the pupil on the return path out of the eye. The amount of light reflected was measured before and after bleaching of the photopigments with a strong light. The difference in these quantities provided a measure of the light absorbed by the photopigments in their unbleached state and presumably that component of the light effective in vision. The differences are greatest for rays passing through the center of the pupil and smallest near the margin of the pupil. The results of these experiments agree quite well with psychophysical measurements of the efficiency of different parts of the pupil made on the same observers (Stiles-Crawford effect). The results support the theory that the cone receptors have directional receiving patterns analogous to those found in other detectors and emitters of radiant energy. 2) Stabilized Images: The effects of the low frequency components of normal eye movements on visual acuity was studied by use of the stabilized image technique. Fine-line and vernier-offset targets were viewed in a stabilized image apparatus which allows the nullification of the effects of eye movements and the elimination of retinal image motion. Controlled retinal image motion was obtained by moving the targets themselves. The effect of complete stabilization was compared with that of 4 cps motion. Amplitudes up to 12 minutes of arc were used. No significant variation in acuity as a function of amplitude was found. Since the range of amplitudes exceeds those found in normal eye movements, doubt is cast on the theory that these motions are helpful to acuity under normal viewing conditions. 3) Electroretinogram. Electrical measurement of incremental thresholds: An electrical analogue of the incremental threshold may be obtained by determining the amount of light necessary to produce a constant small criterion response. In the present experiment this

technique was utilized in dark adaptation and over a wide range of light adaptation levels. Both test and adaptation beams were 52° visual angle and presented to the eye in Maxwellian view. Two durations (0.01 and 0.10 sec.) and three colors of test flash (white, red and blue) were superimposed on a continuous white adaptation field. To obtain a 40 uV criterion response in the dark-adapted eye, approximately 3 log units of light adaptation above the visual absolute threshold were required. Above this level the electrical thresholds were similar to psychophysical thresholds ($\log I$ vs. $\log R$) as functions of color and duration of test flash. A short duration test flash produced a shallower slope of b wave function than a long duration flash. Red light produced a shallower function than white. The functions had positive slopes from approximately 0.5 to 1.0, with the a wave producing consistently shallower slopes than the b wave.

Spectral sensitivity during prolonged dark adaptation. An experiment has been undertaken to investigate the significance of the "break" in the dark adaptation threshold curve of the ERG as a function of time in the dark. In psychophysical experiments the break has usually been considered a transition from cone functioning before the break to rod functioning afterward. However, this same break has also been found in the dark adaptation curves of rod monochromats, casting some doubt on the interpretation. To investigate the problem with the ERG, a pre-exposure of light intense enough to produce the break in the dark adaptation curve was utilized, and spectral sensitivity was measured before and after the break to thirty minutes in the dark. Preliminary data indicate that a break occurs in the threshold data and also in the measures of latency to the b-wave peak. For the b-wave, the break may indicate a transition from dual functioning before, to rod functioning afterwards. This dual functioning seems to be a combination of high red sensitivity plus rod sensitivity. It is planned to investigate the sensitivity of the negative a-wave also during the full thirty minutes of dark adaptation.

Electroretinograms with full retinal stimulation. A translucent contact lens was used to produce diffuse illumination of the whole retina. Electroretinograms obtained with this mode of stimulation were compared with those obtained with a conventional 60° stimulus. A wide range of stimulus energies were used. The principal finding was that full retinal stimulation produced a simplification of the responses. This was evident in the functions relating amplitude, latency, and waveform of the responses to stimulus energy. The results support the theory that the electroretinogram as ordinarily recorded represents responses to two components of the stimulating light: a direct focal component and a stray light component.

Animal visual systems. 1) Spectral sensitivity of the primitive eyes of Limulus polyphemus: Basic mechanisms of vision were studied in eyes of Limulus polyphemus which are relatively primitive and simple compared to the eyes of man. The Limulus possesses ocelli as well as compound eyes. The ocelli are structurally very simple compared to the highly developed compound eyes. The spectral sensitivities of both eyes were studied by recording the electrical

responses to light flashes of controlled wavelength and intensity. It was found that the compound eye had a single spectral sensitivity mechanism while the ocellus had two distinct spectral sensitivity mechanisms, one like that of the compound eye (maximally sensitive in the green part of spectrum) and the other with a maximum sensitivity in the near-ultraviolet. Slow-wave electrical responses of both eyes were studied. The amplitude of the slow waves was plotted as a function of log intensity for various wave lengths of stimulating light and luminance curves were thus obtained. Criterion amplitudes were applied to the luminance curves to give spectral sensitivity functions. The spectral sensitivity is the reciprocal of the relative energy of light required to elicit a constant magnitude response at various wavelengths of light. Since response - luminance functions for the lateral eye are parallel for all wavelengths, the spectral sensitivity function is independent of criterion amplitude. However, the response - luminance functions for the median ocellus are not parallel at all wavelengths. Therefore, the spectral sensitivity function for the median ocellus depends on the criterion used and more than one spectral sensitivity mechanism is involved. Chromatic adaptation was used to enhance the distinction between the two components of the dark-adapted spectral sensitivity of the ocellus. The data are consistent with the hypothesis that the median ocellus contains two photopigments, each probably with its independent receptor: one maximally sensitive in the visible region, and the other with considerably greater sensitivity in the near ultra-violet region. In line with this interpretation the waveform of the ocular response depends upon the wavelength of light. Such a structurally simple eye with two wavelength mechanisms that can be selectively adapted affords the opportunity to study the fundamental mechanisms involved in some detail.

2) Spectral sensitivity of on- and off-responses in the eye of the Frog, *Rana catesbeiana*: Research has continued on the visual system of intact, immobilized *Rana catesbeiana*. The research has been concerned with the relations between the time course of neural activity and spectral mechanisms. Relatively long light flashes have been used to isolate the on- and off-responses. Analysis of ERG data under four different adaptation conditions showed that the on- and off-responses have different spectral sensitivities. Under dark-adapted and low light-adapted conditions the off-responses were relatively more sensitive to shorter wavelength light than the on-responses. With high-energy light flashes and under high light-adapted conditions the spectral sensitivity of the off-response tended to shift toward the longer wavelengths. The short-wavelength sensitivity of the off-response may be related to the blue-sensitive pigment of the green rods. During the course of this research, marked differences in the waveform of the ERG with wavelength of light were noted. These differences may serve as a neural code for color. This hypothesis is being pursued further. At present, the optic nerve discharge and ERG are being recorded simultaneously. A variety of techniques for analyzing their time course to lights of various wavelengths and intensities are being explored. In addition, behavioral techniques are being investigated with this species with the aim of obtaining visual discriminations related to the electrical recording data.

II. Studies of Central Mechanisms.

Computer analysis. The Packard Bell general purpose digital computer continues to be used for the analysis of evoked potential data. It has been supplemented by special purpose equipment for separating low amplitude evoked responses from background noise. This frees it for more elaborate analyses, and several new programs have been written during the past year for this purpose. The computer is now being used for statistical analysis of tabular data and for experiments dealing with the optical properties of the eye, for equipment concerned with the EEG and problem solving as well as, for most of the other experiments described under Studies of Peripheral Mechanisms. The computer is now owned rather than rented by the laboratory and steps are now being taken to increase its memory by an additional 500 words. Although it does not have the advantage of an immediate access memory, the computer will continue to be of high value in future investigations.

Evoked potentials in the visual system: Studies in the adult human. Studies of relations between visual evoked potentials, the electroretinogram, the physical properties of the stimulus, and the behavior of the human observer continue. Experiments dealing with the spectral sensitivity of the electroretinogram and of occipital potential to stimuli at selected positions in the visual field have been completed. The electroretinogram is dominated by scotopic function except when the stimulus is centered on the fovea. In the fovea there is evidence of photopic sensitivity when red stimuli are applied. The occipital response, on the other hand, is dominated by photopic activity for all test colors and positions. Foveal stimulation produces a much larger occipital response than peripheral.

EEG and the development of cortical evoked potentials in human infants. Studies of the electrical responsiveness of the brain of the infant and child have been continued this year with the aim of correlating the development of evoked EEG responses to lights and sounds with other aspects of maturation. The average evoked response to repetitive stimuli has been determined by means of a general purpose digital computer using an averaging program. With increase in age, the onset latency of the flash and click evoked response decreases, most of this decrease taking place in the first months of life. In order to study the early changes with age, a longitudinal study on a group of institutionalized normal infants was begun in December, 1963. Electroencephalograms, psychological and neurological examinations are performed at frequent intervals. Evoked responses during sleep and wakefulness are analyzed separately. A pilot study on the evoked responses to several patterns of the same area and brightness is being done. Electroretinograms of a group of normal infants under two weeks of age are being performed using a contact lens electrode. A group of children with sensory and neurological disorders is being studied. Their patterns and latency of response often differ from normal children; the technique, therefore, holds promise as a diagnostic aid.

Psychophysical investigation of small colored spots.

Previous experiments have shown that small, dim, briefly-presented spots of monochromatic light vary in appearance from flash to flash. Experiments were performed to determine more precisely the apparent hue of such stimuli. Small spots were presented to one eye. Flash duration was always 1 millisecond. The spectral composition and intensity of the stimuli were controlled with filters. A monochromator presented large steady fields to the other eye. The observer controlled the presentation of the stimuli. He first viewed a small spot and then turned on the steady field. He attempted to match the hue of the small spot with the monochromator by adjusting the wavelength setting. A large series of matches of this kind were used to construct frequency distributions of matches. It was found that matches tended to cluster at certain spectral loci. For example, when normally yellow light was used, approximately half of the matches were clustered in the neighborhood of 525 mμ and the other half in the neighborhood of 625 mμ. None of the matches fell in the region of the spectrum from which the light was derived. When other spectral stimuli were used for the small spots the frequency of matches at the two loci varied but the loci themselves remained constant. The results suggest that the stimuli are small enough to stimulate individual classes of color receptors. Further experiments are planned using this class of stimuli. The chief goal of the work is to determine the spectral sensitivity of the receptor systems responsible for the different classes of color experience.

Spectral sensitivity of the turtle in an avoidance conditioning paradigm.

1) An avoidance conditioning paradigm was utilized to measure behavioral spectral sensitivity in the turtle. The animals were trained to withdraw their heads to suprathreshold light to avoid a short-duration shock. They were then subjected to progressively dimmer luminances during extinction. The animal withdrew its head whenever it saw the light and the level of luminance at which it no longer withdrew its head was taken as threshold. Suitable controls were run concurrently during all series. Five animals have been run through an entire series of twelve wavelengths spaced across the visible spectrum. The curves show three well-defined peaks, at 460 - 480 mμ, 650 - 560 mμ, and 640 - 660 mμ. This is of special interest since the ERG recorded under comparable conditions shows only one well-defined peak at 640 - 660 mμ with a shoulder at 575 mμ. Evoked potentials from the optic tectum, the first relay station back of the retina on the other hand, show two well-defined peaks at low criterion heights of response: a red peak near 640 - 660 mμ, and a blue-green peak near 520 - 540 mμ. It would appear that short-wavelength light is weighted more heavily as the stimuli travel toward the central nervous system. In their natural environment turtles are reported to prefer short-wavelength light, the reason presumably linked to orientation to water from land, a possible factor in survival. 2) The luminosity function of the turtle using flicker photometry. An attempt to measure the luminosity function of the turtle eye using flicker photometry in a behavioral situation is in progress. The technique is one in which two lights, one spectral and the other white, are shown in alternation. At an appropriate frequency this array appears to be a flickering light with the hue of the spectral light. By varying the

luminance of the white light the flickering can be made to disappear. A luminosity function is derived by plotting the relative energy of the spectral light necessary to produce a criterion effect as a function of its wavelength. There is a good deal of electrophysiological evidence relating to the spectral sensitivity of the turtle eye, but very little is known how these findings relate to the visual behavior in the turtle. Flicker photometry is well-suited for this task for several reasons. At the human level, luminosity functions derived using flicker photometry are in good agreement with those derived from absolute threshold and heterochromatic matching techniques, but are somewhat more reliable. In addition, luminance levels which are comparable to those used in electrophysiological studies can be used. What is required is the development of a technique in which the turtle will respond differentially to a flickering and a non-flickering light. A two-choice situation has been developed in which the animal must choose between simultaneously presented stimuli. Training with this technique is in progress.

3) Discrimination and free-operant behavior. A free-operant situation involving 20 painted turtles has been completed with the intention of isolating a high and stable response rate that could be used in a discrimination setup with color. In these experiments turtles were confined in a box submerged in water where key pressing by snout could produce an air space that served as reinforcement. A variety of schedules were used. Under CRF, subjects generated from 100 to 200 responses per hour, in some cases up to 300 responses per hour. With ratio schedules, an increase in FR generally produced an increment in response rate up to FR 21. Several FI schedules were examined. Short periods of 6.5, 13, and 26 seconds produced no characteristic scalloping, however in two animals where FI 180 and FI 208 were used, some signs of scalloping or a break-run tendency were observed. VI values of 6.5, 13, 26 seconds produced stable response patterns generating fairly smooth curves. These particular schedules appear to be well-suited as baselines for further studies of sensory processes. It might be noted that the response rate of the turtle is somewhat less than the rates of animals higher on the phylogenetic scale used in free-operant experiments, for example the response rate of the turtle is less than 1/5 of the rat, 1/20 of the pigeon. Because of its slow rate, it is rather difficult to observe local changes in the cumulative curves produced by schedules of reinforcement such as might be the case with moderately high rates of response. However, it is noteworthy that even within the rather restricted ranges investigated effects of different schedules could be seen.

III. Integrative Processes.

The EEG and behavior. Work was continued on the kappa and alpha EEG rhythms and their relation to behavior and other variables. Considerable data have been collected bearing on the relation of kappa and alpha EEG rhythms to problem solving where the problems are presented by one of two sensory modalities: vision and audition. The interest in those relations stems from research reported last year on EEG concomitants of problem solving, when other unexpected results were obtained. One hypothesis that would bridge the discrepant results was that the relation between

these EEG rhythms and problem solving depended on the sensory modality through which information was being received. To test this hypothesis a set of tasks (addition problems) have been constructed which can be presented either visually or aurally and which have been calibrated at three difficulty levels. The problems were automatically presented to individual subjects by a visual display device programmed by punched tape or over a headset from a magnetic tape recorder. The subjects' EEG was recorded and the presence of kappa and alpha rhythms automatically scored. These activities as well as the subject's answers were recorded on a seven channel FM tape recorder. A program was written for the FB 250 computer to analyze the recorded information in various ways. It is hypothesized that differences in kappa rhythm as a function of problem difficulty will appear with auditory, but not visual presentation, while differences will appear in alpha with visual but not auditory presentation. Final data collation on this hypothesis is being pursued. An interesting line of research has been discovered during the course of this investigation. The visual stimuli used for these problems evoked electrical responses which were recorded via standard EEG electrodes and revealed by computer averaging techniques. These results are described below.

Evoked responses to meaningful stimuli. A new procedure which is of considerable interest to understanding evoked responses and of potential use in understanding cognitive processes was introduced. Normal human subjects were instructed to solve simple problems that required perceiving numerical visual stimuli presented in sequence. Other visual stimuli, irrelevant to solving the problems, were a regular part of the stimulus sequence. Responses evoked by these stimuli were recorded with EEG electrodes and the subjects' performance recorded. Differences in evoked responses were found that may not be attributed to the physical stimuli but are related to their meaningfulness in the sense of their task relevance. The stimuli consisted of white luminous figures presented every 3/4 sec. in sequence, e.g. number, blank, number, blank. The subject's task was to say which of the two numbers was numerically smaller, "first" or "second", or whether they were "even". Then the correct answer was presented as a green luminous figure. This sequence constituted a trial lasting 7.5 sec. Each stimulus was a brief darkening followed by the new figure which was maintained until the next stimulus occurred. Ninety trials constituted an uninterrupted run. During each run the numbers (0 - 9) were randomized and the sequence of numerical and non-numerical stimuli was constant. The evoked responses were obtained by bipolar recording from EEG silver disc electrodes fixed to the scalp on the midline, 2.5 cm below the vertex and 2.5 cm above theinion, with the ground electrode attached over the mastoid. Eye movements were monitored by electrodes placed at the external canthi and below one eye. Other kinds of activity were recorded on additional channels at various times. After suitable amplification the evoked responses were revealed by computer averaging of the activity during 90 trials. Each of the stimuli in the trial sequence generally evoked an average response. The responses were consistently different to the number and the blank stimuli. Although the physical intensity of the blanks was approximately 4 times greater, the responses to the numbers were larger. This effect held over a considerable range of

luminances (at least 2000 to 1). A variety of control runs have been made, indicating that the effects are not related to stimulus luminance, eye movements, pupil responses, binocular viewing, alpha EEG activity, or electroretinograms (ERG).

SUMMARY AND CONCLUSIONS:

The eye functions as a light detector but much happens in the first stages of reception to affect the quality of the stimulus. A photoelectric ophthalmoscope has been used to measure the efficiency of different parts of the pupil. The amount of light reflected back out of the eye from different parts of the pupil provides a measure of relative efficiency of the bleaching of photopigments and can be used to assess the amount of light effective in vision. Differences are greatest for light passing through the pupil center and least for light passing through the margin. The results are analogous to psychophysical measures, the Stiles-Crawford effect. Electrical measurements of the eye's response to light has proceeded with the investigation of increment thresholds, i.e. the eye's differential sensitivity to light. Over a wide range of background light at levels corresponding to about 3 log units above visual absolute threshold, the electrical thresholds were similar to psychophysical thresholds. The ERG has also been used to investigate long term dark-adaptation with emphasis on the "red-cone" break. Breaks have been found for various colors, similar to psychophysical functions, for both amplitude and latency measurements. Electroretinograms recorded with full retinal stimulation have shown a simpler waveform when compared to smaller area stimuli. It appears that the ERG as normally recorded reflects both a component of direct focal stimulation as well as a stray light component. Electrical responses from animal eyes have been recorded in both the horseshoe crab and the frog. In the ocelli of Limulus two possible spectral sensitivities have been isolated, one sensitive in the green part of the spectrum and the other sensitive in the near ultraviolet. The two peaks can be influenced by selective adaptation. Spectral sensitivity differences for the on-and off-responses in the frog's ERG indicate that the off-response is more sensitive to short wave-lengths. The difference may serve as a neural code for color and are being followed further along the afferent pathway. Experiments in learning behavior related to color have been done in the turtle as a follow-up to electrical recording done in previous years. An avoidance paradigm has uncovered three peaks in the spectral sensitivity curve which contrasts to the turtle's ERG. Work is being done on both flicker-photometry and free operant conditioning in this same animal. Computer analysis has also been applied to problem solving with kappa- and alpha rhythms. Differences in evoked responses to particular stimuli were found to be related to their meaningfulness in the task under investigation. The computer has been applied to the investigation of evoked potentials in the visual system in human adults, with regard to spectral sensitivity, and in infants for diagnostic purposes.

ARMY RESEARCH TASK REPORT				REPORTS CONTROL SYMBOL CSCRD-6(R2)	
ACCESSION NUMBER 36177			PROJECT, TASK, OR SUBTASK NO. 3A012501B8130805		
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D. C., 20315			2. FUNDING AGENCY Army Medical R&D Command Office of The Surgeon General Washington, D. C., 20315		
3. CONTRACTING AGENCY NA			4. CONTRACTOR AND/OR GOV'T LABORATORY A Walter Reed Army Inst of Rsch Walter Reed Army Medical Center Washington, D. C., 20012 723-1000, Ext 3552		
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Williams, Harold L., Lt Col, MSC, Dept of Clinical and Social Psychology, Division of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012 576-5257 or Interdepartmental Code 198, Ext 5257 See Continuation Sheet 49					
6. TITLE OF: PROJECT <input type="checkbox"/> Analysis of behavior and of mediating mechanisms: TASK <input type="checkbox"/> SUBTASK <input checked="" type="checkbox"/> measurement of performance and decrement of performance (3)					
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964					
8. RESUME (U) Work continued on perceptual encoding, information processing and EEG changes during problem solving. The critical role of language in information processing was elucidated. Longitudinal studies of the learning of grammatical structure of language and the conceptual correlates of verbal behavior were extended to the age period 24-30 months. Several promising criteria for the construction of syntactical models were identified. Other studies show that 5-year olds can learn certain "abstract" concepts previously thought to represent 7- to 8-year levels; the work suggests a redefinition of Piaget's conception of the development of cognitive processes. Studies of acute sleep loss showed that information load interacts with sleep loss to increase performance decrement, and that individual differences in recovery from sleep loss may be related to "payback" of specific stages of sleep. There was evidence that subjects can learn to make differential motor responses to stages of sleep. Studies of visual perception used new apparatus constructed to measure the relative physical sizes of retinal images when viewing targets through an artificial pupil					
9. KEY WORDS Psychology, sociology, behavior, performance, sleep, fatigue, brain, communication.					
10. SUPPORTING PROJECTS Not Applicable					
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet			12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet		
DA FORM 1309R 1 June 63 PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of ____					

ACCESSION NO.
36177

ARMY RESEARCH TASK REPORT

13. PROJECT, TASK OR SUBTASK NUMBER

11 22 23 24 25 26 27 28 29
3 A 0 1 2 5 0 1 B 8 1 3 0 8 0 5

14. DATE OF REPORT (30-33)

30 33 34
0 6 6 4 4

15. SECURITY OF WORK (34)

16. TYPE OF REPORT

35 36 47 48 49 50 51 52 53
3 1 2 6 3

17. SCIENTIFIC FIELD

a. Topical Classific. (56-61)
b. Functional Class (62-64)

56 61 62 64
0 1 0 6 0 5

18. OSD CLASSIFICATION

(65-66)

19. R&D CATEGORY (67)

65 66 67
B R 1

20. CONTRACT NUMBER

11 12 13 14 15 17 18 21 22 26 27
D A

21. GRANT NUMBER

28 29 30 33 34 35 36 38 39 40 41 45 46
D A G

22. ESTIMATED COMPLET. DATES

47 51 52 56 57 61 62 66 67 71
1 C O N T 2 3 4 5

23. PRIORITY (11-14)

24. PROGRAM ELEMENT (15-26)

11 14 15 26
2 6 1 1 2 5 0 1 1

25. CMR&D CODES

27 29 30 32 33 35
N / A

26. CDOG REFERENCE

a. Paragraph No. (36-44)
b. Functional Group (45)

36 39 40 41 42 43 44 45
1 4 1 2 a 6

27. FUNDING

a. Est. Total Cost (11-15)

b. % Spent Intern. (16-18)

" " Extern. (19-21)

c. Total Obligation (22-26)

d. Progrmd. Cur. FY (27-33)

e. " " " +1 (34-40)

f. " " " +2 (41-47)

g. " " " +3 (48-54)

h. " " " +4 (55-61)

i. " " " +5 (62-68)

j. " " " +6 (69-75)

k. Total Man Years of
Effort (76-78)

11 15 16 18 19 21 22 26
1 2
27 28 29 33 34 35 36 40
41 42 43 47 48 49 50 54
55 56 57 61 62 63 64 68
69 70 71 75 76 78

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

- (A) Biersdorf, W. R., Ph.D., Dept of Sensory Psychology, Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012, 576-5260 or Interdepartmental Code 198, Ext 5260 49
- (A) Braine, M. D. S., Ph.D., Dept of Clinical and Social Psychology Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012, 576-5251 or Interdepartmental Code 198, Ext 5251 49
- (A) Clark, W. H., M.A., Dept of Clinical and Social Psychology, Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012, 576-5211 or Interdepartmental Code 198, Ext 5211; University of Maryland Medical School, Baltimore, Maryland 49
- (A) El-Beheri, M. S., Capt, AMSC, Dept of Clinical and Social Psychology, Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012, 576-5211 or Interdepartmental Code 198, Ext 5211 49
- (A) Giesecking, C. F., M.A., Dept of Clinical and Social Psychology, Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012, 576-5251 or Interdepartmental Code 198, Ext 5251 49
- (A) Glanzer, M., Ph.D., Dept of Clinical and Social Psychology, Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012, 576-5211 or Interdepartmental Code 198, Ext 5211; University of Maryland Medical School, Baltimore, Maryland 49
- (A) Hammack, J. T., Ph.D., Dept of Clinical and Social Psychology, Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012, 576-5251 or Interdepartmental Code 198, Ext 5251; Washington School of Psychiatry, 1610 New Hampshire Ave., N. W., Washington, D. C. 49
- (A) Lubin, A., Ph.D., Dept of Clinical and Social Psychology, Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012, 576-5251 or Interdepartmental Code 198, Ext 5251 49
- (A) Morlock, H. C., Jr., Capt, MSC, Dept of Clinical and Social Psychology, Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012, 576-5257 or Interdepartmental Code 198, Ext 5257 49
- (A) Resnick, A., M.S., Dept of Clinical and Social Psychology, Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012, 576-5211 or Interdepartmental Code 198, Ext 5211; University of Maryland Medical School, Baltimore, Maryland 49

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

- (A) Shanks, B. L., M.A., Dept of Clinical and Social Psychology, Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012, 576-5251 or Interdepartmental Code 198, Ext 5251 49
- (A) Wilkinson, R. T., Ph.D., Dept of Clinical and Social Psychology, Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012, 576-5251 or Interdepartmental Code 198, Ext 5251; Washington School of Psychiatry, 1610 New Hampshire Ave., N. W., Washington, D. C. 49
- (A) Williams, J. M., M.A., Dept of Clinical and Social Psychology, Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012, 576-5251 or Interdepartmental Code 198, Ext 5251; Washington School of Psychiatry, 1610 New Hampshire Ave., N.W., Washington D. C. 49

REPORTS. Annual Progress Report, Walter Reed Army Institute of Research, 1 July 1963 - 30 Jun 1964.

Biersdorf, W. R., Ohwaki, S., and Kozil, D. J.: The Effect of Instructions and Oculomotor Adjustments on Apparent Size. Amer. J. Psychol., 76: 1-17, 1963.

Braine, M. D. S.: The Ontogeny of English Phrase Structure: The First Phase. Language, 39: 1-13, 1963.

Braine, M. D. S.: Grammatical Structure in the Speech of Two-Year Olds. Presented to Washington Linguistics Club, Washington, D. C. Proc. Washington Linguistics Club, 1: (1) 11-16 (Summary of Lecture 1963).

Braine, M. D. S.: Learning Language Structures. Paper presented to the American Psychological Association, Philadelphia, 1963.

Braine, M. D. S.: On Learning the Grammatical Order of Words. Psychol. Rev., 70: 323-348, 1963.

Braine, M. D. S.: A Very Young Grammarian. (Review of R. H. Weir, Language in the Crib) Contemporary Psychol., 1964.

Glanzer, M., and Clark, W. H.: Accuracy of Perceptual Recall: An Analysis of Organization. J. verb. Learn. & verb. Behav., 1: 289-299, 1963.

Glanzer, M., & Clark, W. H.: The Verbal Loop Hypothesis: Binary Numbers. J. verb. Learn & verb. Behav., 2: 301-309, 1963.

Glanzer, M., Huttenlocher, J., and Clark, W. H.: Systematic Operations in Solving Concept Problems: A Parametric Study of a Class of Problems. Psychol. Monogr., 77: No. 1, 1963.

Hammack, J. T.: An Experiment in Arousal of Behavior During Sleep. U. S. Army Medical Research and Development Command, Technical Report 16-12, 1964.

Johnson, L. C., Hord, D. J., and Lubin, A.: Response Specificity for Difference Scores and Autonomic Lability Scores. USN Med. Neuropsychiat. Res. Unit, San Diego, Report No. 63-12, 1963.

Loveland, N. T., and Williams, H. L.: Adding, Sleep Loss and Body Temperature. Percept. mot. Skills, 16: 923-929, 1963.

Morlock, H. C., Jr., and Williams, H. L.: The Reinforcement of Performance During Sleep. Presented at Eastern Psychological Association, New York, 1963.

Williams, H. L., Morlock, H. C., and Morlock, J. V.: Discriminative Responses to Auditory Signals During Sleep. Presented at American Psychological Association, Philadelphia, 1963.

Williams, H. L., Hammack, J. T., Daly, R. L., Dement, W. C., and Lubin, A.: Responses to Auditory Stimulation, Sleep Loss and the EEG Stages of Sleep. Electroenceph. clin. Neurophysiol., 16: 269-279, 1964.

Williams, H. L., Morlock, H. C., Jr., Morlock, J. V. and Lubin, A.: Auditory Evoked Responses and the EEG Stages of Sleep. Ann. NY Acad. Sci. 112: Art. 1, 172-181, 1964.

ANNUAL PROGRESS REPORT

Project No.	3A012501B813	Title:	Army Medical Basic Research in Life Sciences
Task No.	08	Title:	Neuropsychiatry
Subtask No.	05	Title:	Analysis of Behavior and of Mediating Mechanisms: Measurement of Performance and Decrement of Performance

Description:

a. Purpose. To analyze the performance of human subjects on tasks which require problem solving, signal detection, symbolic behavior and information processing, and to measure decrement of performance in such tasks under different conditions of load.

b. Approach. Tasks which require sustained performance, at various levels of complexity, are performed by human subjects under normal conditions and during prolonged sleep deprivation or monotony. Methods for modifying performance decrement are studied by investigating the effects of such task variables as pacing, information load, speed load and duration. Electrophysiologic and autonomic correlates of decrement are obtained by concomitant physiologic monitoring. Similar methods are used to study the effects of brain injury. Human capacities for signal detection and discrimination during different stages of sleep are examined by use of instrumental conditioning methods.

c. Symbolic procedures relevant to human problem solving performance are further analyzed by studies of language acquisition, perceptual coding, storage, retrieval and symbolic operations. The repeated measurements used in these studies have required the development of new statistical models for analysis of data.

Progress:

a. Studies of problem solving and data-processing, storage of verbal material, verbal learning, and cognitive processes in language learning.

(1) Problem Solving. The general purpose of the project was to study information processing aspects of problem solving and of other forms of complex performance. These aspects included the encoding and storage of information. During the early phases of the project a variety of problem solving situations were examined. The early exploratory work was followed by an extensive and detailed examination of the solving of concept problems. In the course of carrying this work forward, certain problems in the encoding of information were clarified. This led to a series of experiments on the encoding of perceptual information. The concern with storage of information led to a series of studies on the learning and

remembering of verbal material. This work was further stimulated by findings of the critical role played by language in the encoding of information by human subjects. The work on verbal learning and memory has been concerned in good part with serial position effects in these types of performance.

(a) Work Completed:

1. Information Processing in Problem Solving. An extended series of experiments on the information processing aspects of solving concept problems was completed. These experiments explored the following factors:

(a) Example sign: whether the examples are positive or negative instances.

(b) Concept size: the ratio of the number of relevant dimensions to the total number of example dimensions.

(c) Series complexity: the presence of superfluous information.

(d) Information order: the sequence of examples in series consisting of mixed positive and negative examples.

(e) Storage load: the amount of information that has to be stored at the beginning of a problem.

(f) Selection load: the amount of information required to sort the example dimensions into relevant and irrelevant.

(g) Information rate: the rate at which new information is presented within the example series.

The findings make it possible to control the probability of solution of the concept problems. They also make it possible to specify in considerable detail the systematic operations carried out by the subjects in solving the problems. The operations consist of two distinct stages: 1) specification and storage of dimension values, 2) selection of relevant dimensions on the basis of example information. A general summary of a series of seven experiments carried out under the project has been published in the Psychological Monographs.

2. EEG Concomitants of Problem Solving. The stability of the experimental results from the work on problem solving was great enough to encourage an attempt to correlate specific changes in the EEG with variations in the structure and difficulty of the concept tasks. Experimental work was therefore carried out on the relations between stages of problem solving and characteristics of the EEG output. An extended series of concept problems and control tasks were given to subjects while EEG recordings were made from both the occipital and parietal areas, to measure changes in the output of both alpha and kappa waves. The results show systematic and predictable changes in alpha for each stage of the problem solving. The results for kappa, however, show marked individual differences. These results contrast with the

results of preceding work by Chapman, Armington and Bragdon who, using somewhat simpler mental operations tasks, found that kappa showed marked regularity and predictability while alpha showed relatively weak effects. A report of this study is now in press (Journal of Experimental Psychology).

(b) Work in Progress: EEG Concomitants of Problem Solving.

Further work has been started to analyze the source of the differences in the two types of task mentioned above. The hypothesis currently being tested is that the effects on kappa and alpha are specific to the sense modalities that are involved. Specifically the hypothesis is that problems involving auditory inputs will generate clear effects on kappa while problems involving visual inputs will generate clear effects on alpha. Matched problems have been constructed that can be varied in difficulty and that can be presented either auditorially or visually. These problems are being given to subjects and the effects on both kappa and alpha measured.

(2) Encoding in Perception. The work on problem solving underlined the importance of encoding in the storage and processing of information. In order to study encoding more directly, a series of studies was carried out on the role of encoding in perceptual recall. The main outcome of these studies is a radical simplification of the problem of form perception.

(a) Work Completed:

1. Experiments with Systematically Generated Stimuli of the Type Used in Information Theory Studies. The first series of experiments was carried out to analyze the determinants of the difficulty of perceptual recall of a systematically varied set of stimuli. The stimuli were arrays of eight shapes that were each either black or white. First, the accuracy with which subjects could reproduce these arrays under 1/2 sec. exposure was determined. Then another method involving discrimination between arrays was used to determine the difficulty of the individual stimuli. The discrimination method yielded a similar ranking of accuracy scores. This indicated that the accuracy scores obtained with the reproduction method were not a function of the particular method used. The accuracy scores were then subjected to various types of analysis. An analysis based on information measure showed only partial success in accounting for the difficulty of individual stimuli. Analysis based on gestalt theory was also found to be unsatisfactory. Another type of analysis was constructed, based on the hypothesis that the subjects' perceptual processing includes a covert verbal encoding and that the length of the verbal code determines the difficulty of the stimulus for perceptual tasks. This was labelled the verbal loop hypothesis. Empirically-derived measures based on this hypothesis were shown to account for a major part of the variance in stimulus difficulty. The verbal loop hypothesis is presented as an alternative to gestalt and information theory analyses of organization. The assumption and the findings are highly relevant to the general problem. A paper describing this work has been published in the Journal of Verbal Learning and Verbal Behavior.

2. Experiment on Serial Position Effects in the Storage of Visual Information. The work described above on the role of verbalization in perception suggested that serial position effects in perception may be basically the same as those found in the recall of verbal material. To test this an experiment was carried out in which eight place binary numbers were shown briefly to 12 subjects. The following factors were systematically varied:

(a) Difficulty of the number as defined by its encoding or verbalization length.

(b) Exposure time -- 200, 400, 800, or 1600 milliseconds.

(c) Filled versus unfilled delay period -- half the subjects were required to carry out a recitation task during the delay period, half were not.

The effect of these variables on accuracy of report and on the perceptual serial position curve obtained was measured. All the variables except delay period had a systematic and significant effect on overall performance. Only verbalization or encoding length has a clear and significant effect on the shape of the serial position curve. The results indicate that a simple translation of relations from free recall of verbal material is not possible with this type of perceptual performance. The nature of the relation between the two serial position effects requires further examination.

(3) Cognitive Processes in Language Learning. This work is organized into two complementary sets of investigations. One line of investigation is concerned with the learning of grammatical structure of language. Here, the main goal is to identify the cognitive processes involved in children's acquisition of grammatical structure. The other line of investigation is related to semantic aspects of language development, the ultimate goal being to describe some of the conceptual correlates of verbal behavior, particularly those related to so-called "abstract" thinking.

(a) Work Completed:

1. The Learning of Grammatical Structure. Work on the learning of the structure of artificial languages has continued. An article reporting an earlier series of experiments has now been published in the Psychological Review. Further experiments on the learning of miniature artificial syntactic systems are in progress. These explore the learning of the roles of "function" morphemes (i.e., short, frequently recurring elements such as articles, auxiliary verbs, prepositions, conjunctions, etc.) A paper read at the 1963 APA Convention reported some of these experiments, and showed how the "place-contingency" theory developed in the previous work might account for the learning of some grammatical transforms (i.e., for example, such relations between sentences as active-passive).

(b) Work in Progress: Analysis of the data gathered on the development of English structure in a small sample of children between 18 and 30 months of age has continued. The fairly straightforward data for the first phase of development (18 to 24 months, approximately) were published in a previous year. The data for the period 24-30 months contain about 80 hours of tape-recorded speech as well as fairly extensive written records. Data on one child have been fairly completely analyzed, and for two others analyzed for a part of the time period. Much of the work has been concerned with the development of evaluation criteria for grammars appropriate to a situation where informant judgments of grammaticality are unobtainable. Several promising criteria have been developed, and discussed in a fairly extensive correspondence with other workers.

(c) Work Completed: The Development of Thinking. Experiments have continued in the investigation of conceptual correlates of language development related to "abstract" thinking. The tasks used involve illusory changes of appearance. Illusory size changes are created by a lens or by standard visual illusions, illusory changes of shape are created by partial immersion of rods in water. Work done in previous years has shown that before about seven years of age children do not spontaneously respond differentially to questions like "Which looks bigger?" and "Which is really bigger?" in such tasks. In this laboratory, previous and current work has shown that a majority of children readily learn to respond differentially and correctly to such questions by around age five, and also quickly learn to construe the question "Which is bigger?" as a question about the real rather than the apparent size of the stimuli. The proportion of subjects learning such distinctions is independent of the experimental procedures used so far. A recent experiment shows that the distinction learned on one task transfers to new tasks. These experiments show that "conservation" of size (i.e., a concept of "real" size that is independent of phenomenal transformations), and also conservation of shape exist in a majority of five-year olds. A report on five of these experiments has been submitted for publication, and a report on another one is in preparation.

(d) Work in Progress:

1. The development of "abstract" thinking is also being investigated through study of children's conception of relations of sameness, difference, and similarity. In one experiment, subjects were first taught to respond to an identical pair of figures and to avoid a nonidentical pair. When a reliable first-trial-correct response had been developed, the subjects were tested with generalization problems which involved stimulus-dimensions not used in the initial learning, or in which the positive pair of figures was merely similar (i.e., not identical). A group of three-year-olds and a group of eighteen-month-olds have been run. The results indicate that both groups generalize very widely indeed; even the eighteen-month-old has an extremely general concept of resemblance. The data suggest that the difficulty of detecting similarities is a function of the number of stimulus-dimensions which the subject has to scan in order to detect the similarity. Age differences are probably differences in ability to scan a number of dimensions rather than differences

in the degree of generality of the concept of resemblance. The apparatus and stimuli have recently been used at George Washington University to administer the task to adult rhesus monkeys. Preliminary results suggest that macaques have more difficulty than eighteen-month-old children with generalization problems that involve stimulus-dimensions not used in the initial learning, though not with other kinds of generalization problems; if confirmed this would indicate a less general concept of resemblance in the rhesus monkey than in even very young children, and would represent an important finding.

2. An experiment in progress is designed to explore developmental changes in the concept of similarity after around three years of age. In a procedure similar to that of the preceding experiment, children aged three to six were first taught to respond to a pair of objects which were the same or similar, and to avoid a pair which were different. In a series of generalization problems, the subjects were confronted with a pair of objects similar in appearance but not in function (e.g., a green grape and a green marble), and a pair similar in function but not in appearance (e.g. a grape and a banana); they were asked which were more the same. The main group of subjects were reinforced for choosing the pair that were similar in function; other subjects were reinforced for choosing the pair similar in appearance, and a third group were not differentially reinforced. Results so far indicate that the proportions of subjects in each age-group who learn to respond consistently to the functional similarity are the same as the proportions found in the experiments, described in (c) above, for subjects distinguishing real and phenomenal attributes of objects. These results add further to the definition of the cognitive processes that develop at around this age, and suggests a close connection between the "conservations," and the development of a concept of "type" or "class" which is based on something more than mere phenomenal resemblance.

b. Studies of Sleep and Wakefulness.

(1) Work in Progress:

(a) Experiment 1 - Effects of Acute Sleep Deprivation on Performance. This investigation studies the effects of 1 or 2 nights of sleep loss on performance, and measures correlated changes in electro-physiologic and autonomic variables. The nature of recovery from sleep loss is examined by continuous physiological recording during recovery sleep, and by repeated performance testing during 2 to 4 recovery days. Analyses of recent experiments indicate that both information load and speed load interact with the effects of sleep loss to increase performance decrement. These effects are greatest on work-paced tasks. Analyses of results on a picture-recognition task showed that one night of sleep loss caused decrement in the acquisition of information, but not in retrieval or in long range retention of learned data. Qualitative analysis of verbal

reports by experimental subjects suggested that impaired ability to acquire new information was associated with difficulty in the process of encoding visual displays. Thus the verbal loop hypothesis described earlier in this report is implicated in the studies of acute sleep loss. On the day following one night of recovery sleep, performance on most tasks is almost as efficient as in the baseline period. Furthermore, experimental subjects show no requirement for extra sleep. However, an analysis of EEG stages of sleep over 4 recovery nights shows that sleep loss has differential effects on the duration of the several stages of sleep. The data indicate that when a sleep debt is induced by acute sleep deprivation, Stage 4 is most important in removing the debt. The pay back function for stage 4 appears to be a declining exponential over 4 recovery nights. Individual differences in the amount of stage 4 payback, and in the recovery slope were considerable. Preliminary analysis suggests that subjects who fail to pay back a substantial proportion of the stage 4 debt during the first night of recovery sleep may continue to show performance decrement on the first day after recovery sleep.

(b) Experiment 2 - Discrimination of Auditory Stimuli During Sleep. Research on the ability of human subjects to respond discriminatively to tonal stimuli during natural sleep continued. Two tones were presented on a random schedule throughout the night, and the subject's task was to respond to one only. The tone that was to be responded to was changed from night to night on an ABBA schedule. Sleep was monitored by the EEG. During nights when the subject was given only instructions, performance was generally poor. However when the subject, on subsequent nights, was punished (by abrupt awakening) for failing to respond to the critical tone, performance improved, especially during the "activated" stages of sleep. All subjects were able to switch their responding appropriately as task requirements changed. These data confirmed previous findings and the results of this set of experiments is now being analyzed in preparation for publication.

(c) Experiment 3 - Experimental Analysis of Behavior During Sleep. Recent experiments showed that "depth of sleep" is not a general dimension for ordering the phenomena of sleep. Responsiveness during sleep is a function of:

1. The response class measured.
2. The amount of prior sleep loss.
3. The time of night.
4. The consequences generated by the response.
5. The EEG stages of sleep.

When responses produce no important environmental consequence, response thresholds are very high; when responses prevent severe consequences, response thresholds are very near to those found during waking. Studies of heart rate during sleep indicate that cardiovascular variables may be useful indicators of response thresholds. Linear regression analysis of low on succeeding high heart-rate periods indicates that during the delta stages of sleep homeostatic control functions are either slowed or absent. Thus during stages 3 and 4 (the high-voltage stages) heart rate does not follow the law of initial value. During the emergent activated (dreaming) phase, these regressions resemble those seen during waking. That is, change in rate is a function of the baseline rate, and there is a point of equilibrium below which stimulation, increases, and above which stimulation decreases the heart frequency.

(d) Experiment 4 - Discrimination of the EEG Stages of Sleep. Can the sleeping subject discriminate among stages of sleep, and correctly signal the EEG phase with a motor response? Prior to going to sleep, subjects were instructed to signal the emergent activated (dreaming) stage of sleep by pressing a microswitch. Differential responding was encouraged by associating a conditional stimulus with this phase of sleep. The subject could avoid abrupt awakening by responding within the first two minutes of this period of sleep. Preliminary analysis of results on five subjects shows evidence of learning to respond differentially during the emergent activated stage. Many of these motor responses occur without EEG evidence of awakening.

(e) Experiment 5- Sensory Evoked Responses: Attention and Distraction. It was previously observed, in studies in this laboratory and elsewhere, that cortical evoked potentials to a stimulus diminish when the subject is given a task which diverts him from paying attention to the stimulus. It is questionable, however, whether the diminution is the result of a central attention mechanism in as much as other factors in these experiments (e.g. level of arousal) have not been controlled. A series of studies aimed at studying the effect of attention when other factors were controlled was begun. In the first such study an attempt was made to maintain the subject in the same stage of general arousal under all conditions of the experiment. It had seemed possible that the diminution in evoked response observed during distraction resulted from an increase in alertness. The "attentional" effect, however, was still observed. A further study which will begin shortly is aimed at eliminating the possibility of movement artifacts which might have artifactually raised the evoked potential during those conditions where the subject was attending to the stimulus.

Investigation of the possibility of predicting impairment of performance in vigilance tasks by observing the evoked potential to stimuli preceding the critical signal are also continuing. Pilot studies suggest

that the evoked potential does change slightly prior to the presentation of the critical signal, and knowledge of the changes in the potential brought about by drowsiness and attention provide explanations of the cause of detection failures.

(f) Experiment 6 - The Effects of Incentive and Complexity on Psychophysiological Efficiency. There are certain physiological measures, pulse rate, pulse volume, skin conductance, respiration rate, and muscle tension, which are thought to reflect the general level of arousal or activation of the body. This experiment set out to test this hypothesis by taking these measures under conditions of performance calculated to produce predictably different levels of arousal. These were performance with and without incentive, and performance of a task at two levels of complexity. Two subsidiary questions were:

1. Are these physiological measures unanimous in reflecting changes of arousal within the individual?

2. Is there an interaction between the effects of incentive and task complexity on performance. Results for 12 subjects tested repeatedly for three days are being analyzed.

c. Studies of Brain Injury.

(1) Work in Progress:

(a) A Follow-Up of Japanese B Encephalitis. A collaborative study with the VA has continued on the follow-up of Japanese B Encephalitis. Multivariate analysis of variance, and multiple regression methods were used to analyze patterns of deficit during acute phases of the illness, and patterns of recovery found in the follow-up study. Army tests were available for assessing permanent intellectual functions, temporary deficit during the acute illness, and recovery during follow-up. Wechsler-Bellevue, and various personality tests were administered during hospitalization and the follow-up. Analyses of subscale scores found considerable agreement between Army tests and the Wechsler Adult Intelligence Scale. Data collection for this study has now been completed, and final reports are in preparation.

d. Exploration of Methods of Measurement.

(1) Work in Progress:

(a) The Analysis of Repeated Measurements. Efforts to represent growth and decay processes such as learning and extinction with asymptotic growth curves such as the logistic were largely unsuccessful. Certain relatively simple curves such as the Mitscherlich were found to

fit empirical data averaged over groups of subjects, but individual subjects showed consistent deviations from theoretical values. Theoretical and empirical studies of the law of initial value have been more successful. Equations were developed which combined the effects of initial level, stimulation and habituation. Analyses showed that the law of initial values may apply to autonomic variables for which there is direct neural negative feed back, but not to variables where there is no such feedback mechanism.

e. Size and Distance Perception. An experiment has been completed measuring size and distance perception of subjects seeing lighted targets within a blacked out vision tunnel. Instructions to the subjects were varied and convergence of the eyes was measured. Under some stimulus conditions, distance perceptions significantly reversed from true physical distance were obtained, indicating that size judgments were not dependent on distance judgments of the same targets.

An experiment has been designed and apparatus constructed to measure the relative physical sizes of retinal images when viewing targets through an artificial pupil. When using an artificial pupil, retinal image size is dependent on the stage of accommodation. By the use of a binocular comparison technique with an artificial pupil before one eye only, and varying accommodative stimuli; it will be possible to measure the magnitude of this effect. The results will be of interest both from the viewpoint of physiological optics and also as a control technique in experiments on size perception.

Summary and Conclusions:

a. Studies of problem solving and the organization of perception clarified the critical role played by language in the encoding of information by human subjects, and identified a number of problem characteristics which control the probability of solution of concept problems. Certain EEG patterns were found to correlate with variations in the structure and the difficulty of concept tasks. An important outcome of these studies of perception and concept attainment has been a radical simplification of the problem of perceptual organization.

b. Studies of the development of language and thinking in children produced a number of promising criteria for the evaluation of grammars, and showed that when adequate techniques of training are used, children can learn concepts such as "conservation," relations of similarity and difference, and differences between real and apparent size at about age 5. The work suggests a redefinition of Piaget's conception of the cognitive processes that develop at around this age.

c. Studies of sleep and wakefulness explored task variables such as speed load which interact with sleep loss, found evidence that impaired recall functions during sleep loss are due to difficulty in the process of encoding information, and showed that behavior (including discrimination) can be obtained from the sleeping subject.

d. New studies of sensory evoked responses from the human scalp showed that these changes in electrical potential are affected by attention and vigilance.

e. Studies of methods of measurement and exploration of optimal test scoring methods continued.

ARMY RESEARCH TASK REPORT				REPORTS CONTROL SYMBOL CSCRD-4(R2)	
ACCESSION NUMBER 36178			PROJECT, TASK, OR SUBTASK NO. 3A012501B8130911		
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D. C., 20315			2. FUNDING AGENCY Army Medical R&D Command Office of The Surgeon General Washington, D. C., 20315		
3. CONTRACTING AGENCY NA			4. CONTRACTOR AND/OR GOV'T LABORATORY A Walter Reed Army Inst of Resch Walter Reed Army Medical Center Washington, D. C., 20012, 723-1000, Ext 3552		
5. PRINCIPAL & ASSOC. INVESTIGATOR/PROJECT OR ACTION OFFICER (P) Glinos, Andre D., M. D., Dept of Cellular Physiology Div of Bas Surg Resch, WRAIR, WRAMC, Washington, D. C., 20012 576 5284 or Interdepartmental Code 198, Ext 5284 See Continuation Sheet 49					
6. TITLE OF: PROJECT <input type="checkbox"/> TASK <input type="checkbox"/> SUBTASK <input checked="" type="checkbox"/> Cell growth and regeneration (U)					
7. DATE OF REPORT		DAY 30		MONTH June YEAR 1964	
8. RESUME (U) Work on the mechanisms controlling cellular growth and regeneration has continued utilizing the tissue culture system previously described. The kinetics of post-irradiation cell growth and regeneration in this system indicated (a) that cell division is controlled by an intracellular constituent situated outside the genetic apparatus and (b) that the concentration of this constituent varies for a given cell population, being maximum during the logarithmic and minimum during the stationary phase. To identify and define in molecular terms this constituent, extensive analysis of the metabolic activity of the cells in the logarithmic and stationary phases was undertaken. The results strongly suggest that this constituent is located somewhere in the RNA-protein complex and that energy metabolism is among the factors regulating its synthesis. Closer identification of the constituent(s) controlling cell division and its relationship to energy metabolism are the objects of further studies aiming at the eventual chemical isolation and pharmacological use of this constituent in infection, radiation injury, wound healing and transplantation where cell division and proliferation have been shown to be the determining factors.					
9. KEY WORDS Cell division control, RNA, protein, energy, infection, radiation, wound healing, transplantation.					
10. SUPPORTING PROJECTS Not Applicable					
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet			12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet		
FORM 1309R DA 1 June 63 PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of _____					

ACCESSION NO.
36178

ARMY RESEARCH TASK REPORT

13. PROJECT, TASK OR
SUBTASK NUMBER

11 22 23 24 25 26 27 28 29
3 A 0 1 2 5 0 1 B 8 1 3 0 9 1 1 1 1

14. DATE OF REPORT (30-33)

30 33 34
0 6 6 4 4

15. SECURITY OF WORK (34)

16. TYPE OF REPORT

35 36 47 48 49 50 51 52 53
3 1 2 6 3

17. SCIENTIFIC FIELD

a. Topical Classific. (56-61)
b. Functional Class (62-64)

56 61 62 64
0 1 0 6 0 0 1 1

18. OSD CLASSIFICATION

(65-66)

19. R&D CATEGORY (67)

65 66 67
B R 1

20. CONTRACT NUMBER

11 12 13 14 15 17 18 21 22 26 27
D A 1 1 1 1 1 1 1 1

21. GRANT NUMBER

28 29 30 33 34 35 36 38 39 40 41 45 46
D A 1 1 1 1 1 1 1 1 G 1 1 1 1

22. ESTIMATED COMPLET.
DATES

47 51 52 56 57 61 62 66 67 71
1 2 3 4 5

23. PRIORITY (11-14)

24. PROGRAM ELEMENT
(15-26)

11 14 15 26
2 6 1 1 2 5 0 1 1

25. CMR&D CODES

27 29 30 32 33 35
N / A 1 1 1 1

26. CDOG REFERENCE

a. Paragraph No. (36-44)
b. Functional Group (45)

36 39 40 41 42 43 44 45
1 4 1 2 a 6

27. FUNDING

a. Est. Total Cost (11-15)

b. % Spent Intern. (16-18)

" " Extern. (19-21)

c. Total Obligation (22-26)

d. Progrmd. Cur. FY (27-33)

e. " " " +1 (34-40)

f. " " " +2 (41-47)

g. " " " +3 (48-54)

h. " " " +4 (55-61)

i. " " " +5 (62-68)

j. " " " +6 (69-75)

k. Total Man Years of

Effort (76-78)

11 15 16 18 19 21 22 26
1 2 2 2
27 28 29 33 34 35 36 40
41 42 43 47 48 49 50 54
55 56 57 61 62 63 64 68
69 70 71 75 76 78

DA FORM 1309R
1 Jun 63

Previous Editions are Obsolete

Page 2 of

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

- (A) Greer, R. B., III, Capt, MC, Dept of Cellular Physiology
Div of Bas Surg Rsch, WRAIR, WRAMC, Washington, D. C., 20012
576-5179 or Interdepartmental Code 198, Ext 5179 49
- (A) North, H. H., B. S., M. T., Dept of Cellular Physiology
Div of Bas Surg Rsch, WRAIR, WRAMC, Washington, D. C., 20012
576-5307 or Interdepartmental Code 198, Ext 5307 49
- (A) Werrlein, R. J., B. S., Dept of Cellular Physiology
Div of Bas Surg Rsch, WRAIR, WRAMC, Washington, D. C., 20012
576-5307 or Interdepartmental Code 198, Ext 5307 49
- (A) Hargrove, D. D., SP4, Ph. D., Dept of Cellular Physiology
Div of Bas Surg Rsch, WRAIR, WRAMC, Washington, D. C., 20012
576-5307 or Interdepartmental Code 198, Ext 5307 49
- (A) Hajkowski, S. D., SP5, Dept of Cellular Physiology
Div of Basic Surg Rsch, WRAIR, WRAMC, Washington, D. C., 20012
576-5179 or Interdepartmental Code 198, Ext 5179 49
- (A) Burnette, D. W., PFC, M. S., Dept of Cellular Physiology
Div of Bas Surg Rsch, WRAIR, WRAMC, Washington, D. C., 20012
576-5179 or Interdepartmental Code 198, Ext. 5179 49

REPORTS. Annual Progress Report, Walter Reed Army Institute of Research, 1 July 1963 - 30 June 1964.

Glinos, A. D., and North, H. H.: Cellular Growth and Tissue Radiosensitivity: Cell Studies in Vitro and General Concepts. Trans. N. Y. Acad. Sci., 26:145-158, 1963.

Greer, R. B., and Glinos, A. D.: Oxygen Utilization by Cultures of Mammalian Cells -- A New Analytic Method. Fed. Proc., 23: 574, 1964. (Abstract)

ANNUAL PROGRESS REPORT

Project No. 3A01250B813 ARMY MEDICAL BASIC RESEARCH IN LIFE SCIENCES

Task No. 09 Physiology

Subtask No. 11 Cell growth and regeneration

Description:

The available evidence indicates that cell division and proliferation is the determining factor in the following situations of Medical Military interest: (a) infection, (b) radiation injury, (c) wound healing, and (d) transplantation.

(a) In Infection it has been well known for years that the first defense response of the host is the division and proliferation of the stem cells of polymorphonuclear leukocytes capable of phagocytosis. The recent revolution in thinking regarding infection consists of the demonstration that the immune process is also one of cellular division and proliferation, involving a complex of cells capable of producing antibody, in response to antigenic stimulation.

(b) In Radiation it has been well known for years that the injury is mainly located in the rapidly dividing and proliferating cells. Recent work has shown that interactions between changes induced at the time of irradiation and the processes of post-irradiation cellular growth and division account for the extensive injury of proliferating cells. Such injury when located in the cells discussed in the previous paragraph is in turn responsible for the high susceptibility to infection of the irradiated host.

(c) In Wound Healing regeneration and repair of lost tissue depends on the controlled cellular division and multiplication of epithelial and mesenchymal elements, the latter being responsible for the elaboration of the intercellular matrix substances such as collagen.

(d) In Tissue Transplantation the response of the host and the fate of the graft is determined by the extent of division and proliferation of cells capable of responding to tissue antigens in a manner similar to the one described in par. (a) for infection.

Knowledge of the mechanisms controlling cell division would seem therefore to be the most essential prerequisite for the eventual mastery and control of all clinical situations related to infection, injury, wound healing and transplantation and has accordingly been defined as the objective of this subtask.

Progress:

Using irradiation as the noxious agent the kinetics of post injury cellular growth and regeneration were investigated in a mammalian tissue culture system approximating body tissues in situ as previously described (see Progress Report 1 July 1962 - 30 July 1963). The following results were obtained when the post-irradiation growth kinetics of cells originating in the logarithmic and stationary phases of the cell population cycle were compared:

1. With respect to their ability for indefinite post-irradiation division and clone formation stationary and logarithmic origin cells were equally sensitive.

2. An inverse linear logarithmic relationship to dose was described with respect to the limited post irradiation divisions completed by cells rendered incapable for clone formation.

3. The linearity of this relationship was demonstrated for both logarithmic and stationary origin cells but for the same dose, logarithmic origin cells completed a significantly higher number of divisions than stationary origin cells.

The results in 1 were considered to indicate that the injury to the genetic apparatus of logarithmic and stationary origin cells was identical.

The results in 2 were considered to indicate the dose-dependent inactivation or impairment of synthesis of a constituent, located outside the genetic apparatus, with a key role in the control of cell division. A certain initial concentration of this key constituent would enable the cells to undergo a certain number of divisions before dilution of this constituent below a minimum essential level.

The results in 3 were considered to indicate that the initial concentration of this key constituent is higher in logarithmically growing cells than in cells in the stationary phase.

This last conclusion can be brought to further experimental verification and to be used as an opening wedge for the identification of the division controlling constituent by monitoring the mean concentration per cell of known key molecular classes during the transition of a cell population from the logarithmic to the stationary phase.

If during this transition period the concentration of any of these known molecular classes is found to decrease in a consistent and meaningful fashion there would be a high probability that the postulated regulation of cell division is associated with this particular molecular class.

To this end suspension cultures of the L strain mouse fibroblasts were used in twenty experiments of a design and scope similar to the one illustrated in Table I. Thus cell growth kinetics (columns B, C, D), protein, RNA and DNA concentration (columns E, F, G, H) and energy metabolism (columns I, J, K, L) were simultaneously investigated during the transition from the logarithmic to the stationary phase. In Table I it can be seen that for the first 48 hours growth was nearly logarithmic but with a slowly declining growth rate as represented by the figures of column C for the relative cell number and of column D for the percent of cells in actual mitotic division at 24 and 48 hours. The low mitotic figure at 0 time is due to the lower than incubation temperature and the other manipulations necessary for setting up the experiment to which the culture was exposed just prior to this time.

Paralleling this declining growth rate it can be seen that the average concentration of protein and RNA per cell also decreased from 0 to 48 hours while the concentration of DNA remained constant (columns E, F, G). This finding of an early decrease of the cellular protein and RNA concentration obtained through chemical determinations (the method of Oyama and Eagle Proc. Soc. Exp. Biol. Med., 91: 305, 1956, for protein, and of Scott, Fraccastoro and Taft, J. Cytochem. Histochem., 4: 1, 1956 for RNA) is further supported by physical measurements of mean cell volumes recorded in column H and obtained by sizing the cells with the Coulter electronic counter. These data clearly show that as it would be expected, the decrease of cellular protein and RNA during the first 48 hours is also reflected in a significant reduction of the average cell volume within this time. Since moderate but significant changes in growth rate and in protein, RNA and cellular volume were already evident at 48 hours and in order to monitor as closely as possible the transition to the stationary phase expected to follow, all determinations beyond the 48th hour were carried out at 6 hour intervals. While the cell count and relative cell number (columns B and C) show that a significant number of cells did undergo division between 48 and 54 hours, the actual number of dividing cells seen at the 54th hour (column D), i.e. the end of the 6 hour period, shows a dramatic decline. This finding of a rather abrupt inhibition of cell division, obtained through microscopic observations, is further supported by the chemical determinations of DNA (method of Scott Fraccastoro and Taft, J. Cytochem. and Histochem. 4: 1, 1956) recorded in column G. Inhibition of cell division would obviously be reflected also as inhibition of DNA synthesis and the latter would lead to elimination from the population of cells either in the process of synthesis or having already synthesized new DNA. The elimination of these cells from the population would in turn be reflected by a significant decline of the average DNA value per cell, and this is precisely what a comparison of the DNA data of column G at 48 and 54 hours, indicates.

Protein, RNA and cell volume data (columns E, F and H) at 54 hours show that the gradual decrease previously observed continues

but with no dramatic change as the one described with respect to mitotic index and DNA.

The cell count, relative cell number and mitosis incidence data (columns B, C, D) at 60, 66 and 72 hours show that the culture has reached the stationary phase which is characterized by further moderate decreases in cell protein, RNA, and volume while DNA shows no further significant change (columns E, F, H, G).

Thus, the concept that cell division is controlled by an intracellular non-genic constituent the concentration of which is high during the logarithmic and low during the stationary phase has been further substantiated. Furthermore, these metabolic studies place the location of this constituent somewhere in the protein-RNA complex since decline of the concentration of these molecular species was shown to precede the inhibition of DNA synthesis and of cell division characteristic of the stationary phase.

While this stationary phase was induced by omitting renewal of the medium and subculture of the cell population, the specific limiting reactions responsible for the inhibition of DNA synthesis and of cell division are not known. In order to explore the role of energy producing reactions in this connection, glucose uptake and lactate production (columns I, J, K, and L) were also determined in the type of experiments illustrated in Table I. It can be seen that while up to the 54th hour, glucose uptake and lactate production per cell (columns J and L) were high and relatively constant, a rather abrupt decrease in both occurred with the advent of the stationary phase at 60 hours (as expressed by the cell count and relative cell number data; columns B and C). There was a further decrease of glucose uptake and lactate production at the 66 and 72 hours intervals of the stationary phase. Since the glucose of the medium was shown to be virtually depleted at 60 hours (column I) it would seem reasonable to assume that it is this depletion which caused the slowing down of the energy producing glycolytic reactions reflected in the values of glucose uptake and lactate production (columns J and L) and which in turn led to inhibition first of protein-RNA and then of DNA synthesis (columns E, F, and G) and finally to inhibition of cell division (columns B, C, and D), i.e. to the stationary phase. While such a notion would seem to be consistent with the generally accepted idea that glycolysis is a characteristic metabolic feature of rapidly growing cells it would also have to take into account Zwartouw and Westwood's proposal that glycolysis and growth can readily be dissociated by manipulating the pH of the culture (Brit. J. Exp. Path. 39:529, 1958). According to these authors at high pH values glycolysis is stimulated and growth inhibited while the opposite situation prevails at a low pH.

In the experiment summarized in Table I a bicarbonate buffer system and a CO₂ incubator were used in order to avoid any pH effects. The gas phase used was 5% CO₂ in air from column M it can

be seen that under these conditions no significant pH changes occurred during the experiment. In order to test the Zwartouw and Westwood proposal, a new method involving O_2 uptake measurements in addition to cell counts, glucose uptake and lactate production in cell cultures was developed. By carrying out these measurements at different pH values kept constant for each experiment, relationships between growth, glycolysis and respiration can readily be investigated as shown in the two experiments summarized in Tables II and III.

The experiment summarized in Table II was conducted at a pH of 7.15 and is in all respects quite comparable to the experiment of Table I. The similarity of the results obtained can be seen in the duration of the active growth phase -- 48 to 56 hours in both experiments; in the average levels of glucose uptake and lactate production per cell per minute during this period of active growth (units are $mg \times 10^{-10}$) -- glucose uptake: 2.83, lactate production: 1.39, in the experiment of Table I, and glucose uptake: 2.43, lactate production: 1.30, in the experiment of Table II; in the sudden inhibition of growth at 60 hours -- relative cell number: 0.97 in Table I and 0.38 in Table II; in the marked decrease of the levels of glucose uptake and lactate production at this time -- glucose uptake: 1.15, lactate production: 0.78 in Table I, and glucose uptake: 1.38, lactate production: 0.86 in Table II; and finally in the fact that when this parallel inhibition of growth and glycolysis occurred at 60 hours the medium glucose was virtually exhausted -- 61.5 mg/L of glucose in Table I and 14 mg/L in Table II. The additional information obtained in the experiment of Table II concerns the fact that O_2 uptake per cell decreased gradually up to 48 hours and abruptly at 60 hours when growth and glycolysis were also inhibited and that this decrease could not be attributed to exhaustion of O_2 dissolved in the culture medium.

The experiment summarized in Table III was conducted at a pH of 7.41 and the results obtained are in all respects strikingly different from the other two experiments just discussed. Thus, the duration of the growth period was 24 instead of 48 hours; the average level of glucose uptake was 5.0 instead of 2.43 -- 2.83; lactate production was 3.02 instead of 1.30 -- 1.39; inhibition of growth was manifested as early as 36 hours when the relative cell number reached the value of 0.95 but this inhibition was not coupled with marked decreases of glucose uptake and lactate production which remained at the respective levels of 4.08 and 1.35; finally when this inhibition of growth at 36 hours occurred, the medium glucose was not exhausted, its concentration remaining at 129 mg/L. Also O_2 uptake per cell did not decrease but on the contrary increased gradually reaching its maximum value at 36 hours, when growth was inhibited.

Comparison of the results obtained in the experiments of Tables I and II on one hand and Table III on the other, not only supports Zwartouw and Westwood's idea that at high pH values growth and glycolysis are dissociated but suggests that this dissociation extends

also to respiration as expressed by the O₂ uptake data.

It is therefore clear that if energy availability is one of the limiting factors with respect to the synthesis of the RNA-protein complex regulating cell division this relationship is by no means a simple one. Its elucidation must await the outcome of further work aiming both at a more specific definition of the RNA-protein constituent(s) regulating cell division and its relationship to the metabolism of energy.

Summary and Conclusions:

Work on the mechanisms controlling cellular growth and regeneration has continued utilizing the tissue culture system previously described. The kinetics of post-irradiation cell growth and regeneration in this system indicated (a) that cell division is controlled by an intracellular constituent situated outside the genetic apparatus and (b) that the concentration of this constituent varies for a given cell population, being maximum during the logarithmic and minimum during the stationary phase. To identify and define in molecular terms this constituent extensive analysis of the metabolic activity of the cells in the logarithmic and stationary phases was undertaken. The results strongly suggest that this constituent is located somewhere in the RNA-protein complex and that energy metabolism is among the factors regulating its synthesis.

Closer identification of the constituent(s) controlling cell division and its relationship to energy metabolism are the objects of further studies aiming at the eventual chemical isolation and pharmacological use of this constituent in infection, radiation injury, wound healing and transplantation where cell division and proliferation have been shown to be the determining factors.

A	B	C	D	E	F	G	H	I	J	K	L	M
Time	Cell Count ml	Relative Cell No. Period	Mitosis No/100 Cells	Protein mgm x 10 ⁻⁸ per cell	RNA mgm x 10 ⁻⁸ per cell	DNA mgm x 10 ⁻⁸ per cell	Mean Cell Volume u ³ /cell	Glucose mgm/ml	Glucose Uptake ugm x 10 ⁻⁷ cell/min	Lactate mgm/ml	Lactate Product. ugm x 10 ⁻⁷	pH
0	365,000	--	0.25	444.98	52.96	26.69	1465.5	888	--	90		7.32
24	818,000	2.24	1.75	275.06	40.64	28.12	1365.0	680	2.44	231	1.65	7.27
48	1,482,000	1.81	1.45	343.69	38.69	26.32	1268.5	297	2.31	400	1.02	7.21
54	1,660,000	1.12	0.35	316.26	36.14	15.06	1122.5	84	3.76	485	1.50	7.18
60	1,614,000	0.97	0.20	321.21	20.00	10.97	1181.0	16.5	1.15	531	0.78	7.13
66	1,674,000	1.04	0.35	313.62	32.24	11.57	1210.0	8.0	0.14	588	0.96	7.13
72	1,757,000	1.05	0.25	250.85	31.90	16.12	1152.0	8.0	0.00	575	-0.08	7.17

TABLE I

Gas phase - Air + 3% CO₂
pH - 7.15 ± 0.03

Time in hours	Cell count per ml. x 10 ⁵	Relative cell increase per 12 hr. period	O ₂ conc. in Solution Vol. %	O ₂ uptake per cell ml x 10 ⁻¹⁰ per minute	Glucose mg/L	Glucose uptake per cell mg x 10 ⁻¹⁰ per minute	Lactate mg/L	Lactate production per cell mg x 10 ⁻¹⁰ per minute
0	4.46				866		117	
12	6.65	1.49	0.25	1.04	768	2.45	178	1.51
24	10.58	1.59	0.14	0.85	619	2.41	281	1.61
36	13.03	1.23	0.09	0.75	418	2.37	376	1.11
48	15.22	1.17	0.17	0.54	164	2.49	477	0.98
60	14.97	0.98	0.23	0.04	14	1.38	571	0.86

TABLE II

Gas phase - Air only
pH - 7.41 ± 0.01

Time in hours	Cell Count per ml. $\times 10^5$	Relative cell increase per 12 hr. period	O ₂ conc. in solution Vol. %	O ₂ uptake per cell $\text{ml} \times 10^{-10}$ per minute	Glucose mg/L	Glucose uptake per cell $\text{mg} \times 10^{-10}$ per minute	Lactate mg/L	Lactate production per cell $\text{mg} \times 10^{-10}$ per minute
0	5.07				912		101	
12	6.72	1.32	0.14	1.04		4.73		3.50
					711		250	
24	9.57	1.29	0.18	1.28		5.28		2.64
					402		405	
36	9.00	0.95	0.19	1.78		4.08		1.35
					129		495	

TABLE III

ARMY RESEARCH TASK REPORT		REPORTS CONTROL SYMBOL CSCRD-6(R2)
ACCESSION NUMBER 36197		PROJECT, TASK, OR SUBTASK NO. 3A013001A8140101
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D. C., 20315	2. FUNDING AGENCY Army Medical R&D Command Office of The Surgeon General Washington, D. C., 20315	
3. CONTRACTING AGENCY NA	4. CONTRACTOR AND/OR GOV'T LABORATORY A Walter Reed Army Inst of Rsch Walter Reed Army Medical Center Washington, D. C., 20012 723-1000, Ext. 3552	
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P)Mertz, Walter, M. D., Division Biochemistry WRAIR, WRAMC, Wash. D. C. 20012 576-3528 or Interdepartmental Code 198, Ext. 3528 See Cont. Sheet		
6. TITLE OF: PROJECT <input type="checkbox"/> TASK <input type="checkbox"/> SUBTASK <input checked="" type="checkbox"/> Biochemical action of trace substances--effects of trace metals on hormone and enzyme activity (U)		
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964		
8. RESUME (U) The catalytic role of chromium (III) in the interaction of insulin with its receptor site in membranes was further investigated using epididymal fat tissue <i>in vitro</i> as test system. The results show the efficiency of this catalytic role depends on the state of oxidation as well as the nature of the ligands coordinated to the remaining positions on the chromium ion. Various <i>in vivo</i> systems were developed to test the concept of essentiality of chromium (III). Preliminary data suggest a beneficial role of chromium in various situations. In clinical studies with chromium supplementation, a number of subjects showed significant improvement of glucose metabolism with supplemental chromium. Biochemical changes of manganese toxicity in rats were studied. Small, but significant changes were detected in glucose metabolism as well as in the respiration of liver and brain slices.		
9. KEY WORDS Insulin, chromium, trace metals, diabetes, manganese, glucose metabolism.		
10. SUPPORTING PROJECTS Not applicable.		
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet	12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet	
FORM 1309R 1 June 63 PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of 1		

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

(A) Roginski, E. A., Dept Biol Chem, Div Biochemistry,
WRAIR, WRAMC, Washington, D. C., 20012
576-3527 or Interdepartmental Code 198, Ext 3527 49

(A) Glinemann, W. H., Capt, MC, Dept Metabolism,
Div Medicine, WRAIR, WRAMC, Wash. D. C. 20012
576-3573 or Interdepartmental Code 198, Ext 3573 49

(A) Reba, R. C., Major, MC, Dept Isotope Metabolism,
Div Nucl Med, WRAIR, WRAMC, Wash. D. C. 20012
576-3305 or Interdepartmental Code 198, Ext 3305 49

(A) Pentschew, A. J., M. D., Neuropathology Branch,
Armed Forces Inst Path, WRAMC, Wash. D. C. 20012
576-3400 or Interdepartmental Code 198, Ext 3400 49

REPORTS. Annual Progress Report, Walter Reed Army Institute
of Research, 1 July 1963 - 30 June 1964.

ANNUAL PROGRESS REPORT

Project No. 3A013001A814/ (1) Title: In-House Laboratory Initiated R&D

Task No. 01 Title: In-House Lab Initiated R&D

Subtask No. 01 Title: Biochemical action of trace substances--effects of trace metals on hormone & enzyme activity

Description: In fat, a muscle tissue from chromium-deficient rats, the oxidation of Cl^{14} labeled glucose to Cl^{14}O_2 is increased only by addition of unphysiologically high doses of insulin. Addition of milligram amounts of chromium significantly increases the response of tissue to small insulin doses. This system is well suited to test the biological activity of various chromium compounds.

Progress: A number of complexes prepared by Dr. Rollinson (U of Md) were compared. The results thoroughly indicate that a certain degree of oxidation is correlated with maximum biological activity. The exact number cannot be determined at present, but experiment is being assembled to exactly define the structure of the compound. The role of the additional ligands on the metal was investigated in the same test system, by coordinating chromium with various ligands of biological interest. The results indicate that certain amino acid-chromium chelate are superior in bioactivity to all other compounds tested so far. These studies are expected to define the mechanisms by which the catalytic function of chromium on the action of insulin, as well as other hormones is brought about.

A beneficial effect of chromium on growth and longevity has been demonstrated only by one laboratory, specially equipped to maintain trace element-free conditions. We were unable to duplicate these results at their Institute, due to environmental contaminations which cannot be controlled in this situation. Therefore experiments were set up in which animals on chromium deficient and supplemented diets were subject to measured amounts of various stresses. Under these conditions, beneficial effects of chromium on growth, survival and rate of recovery were detected. These experiments are still in progress. First, incomplete results of in vitro studies suggest that these effects related to a role of chromium in protein metabolism.

Clinical studies (with Dr. W. Glinsmann, Dept Metab) showed dramatic but unsustained effects of chromium administration in human diabetes. With a different way of dosing the metal, so far applied only to two patients, the first diabetic was essentially normalized throughout the period of chromium treatment. The second case is now still under treatment. Simultaneous studies of these patients with chromium⁵¹ established preliminary rates of chromium metabolism and excretion (with Dr. Richard Reba).

Chromium supplementation did not further improve normal or better than normal glucose tolerance of young, healthy volunteers, but it produced sustained, insignificant improvement in a number of older individuals. Since the doses given are not much above the estimated dietary intake, these findings suggest the existence of chromium deficiency in older people.

Previous studies by Dr. Pentchew, AFIP, had shown that the histopathological changes in the subcortical centers in manganese-poisoned rats were not due to a local mutation by the metal, but most probably caused by an indirect mechanism. Therefore carbohydrate metabolism and, more specifically the oxidation of carbohydrate by liver stores were studied in these animals. The experiments are still in progress; the first results have demonstrated increased fasting blood glucose levels and increased O₂ consumption of liver in the Mn-poisoned animals, indicating that the brain damage is preceded and possibly caused by a disturbance in carbohydrate metabolism.

Summary and Conclusions:

The catalytic role of chromium (III) in the interaction of insulin with its receptor site in membranes was further investigated, using epididymal fat tissue *in vitro* as the test system. The results show that the efficiency of this catalytic role depends on the state of oxidation as well as the nature of the ligands coordinated to the remaining positions on the chromium ion.

Various *in vivo* systems were developed to test the concept of essentiality of chromium (III). Preliminary data suggest a beneficial role of chromium for rats in various situations. The mechanism of this action is under investigation.

Clinical studies with chromium supplementation of normal humans and diabetics were continued. A number of persons were shown to respond to the treatment with significant improvement of glucose metabolism.

Biochemical changes of manganese toxicity in rats are studied in collaboration with Dr. A. Pentschew, AFIP. Small, but significant, changes were detected in glucose metabolism as well as in the respiration of liver and brain slices. These changes can be correlated with the anatomical and neurological lesions experimentally produced.

ARMY RESEARCH TASK REPORT		REPORTS CONTROL SYMBOL CSCRD-6(72)
ACCESSION NUMBER 36199	PROJECT, TASK, OR SUBTASK NO. 3A013001A8140104	
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D. C., 20315	2. FUNDING AGENCY Army Medical R&D Command Office of The Surgeon General Washington, D. C., 20315	
3. CONTRACTING AGENCY NA	4. CONTRACTOR AND/OR GOV'T LABORATORY A Walter Reed Army Inst of Resch Walter Reed Army Medical Center Washington, D. C., 20012 723-1000, Ext 3552	
5. PRINCIPAL & ASSOC. INVESTIGATOR/SUBJECT OR ACTION OFFICER (P) Wampler, S. N., Capt, VC, Dept of Food Radionuclides, Div of Veterinary Medicine, WRAIR, WRAMC, Washington, D. C., 20012 074-3646 or Interdepartmental Code 198, Ext 3646		
6. TITLE OR PROJECT <input type="checkbox"/> TASK <input type="checkbox"/> SUBTASK <input checked="" type="checkbox"/> Neutron-induced radioactivity in subsistence (U)		
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964		
8. SUMMARY (U) The major objective of the project is to investigate the problems of neutron-induced radioactivity in subsistence items. The subtask is a preliminary investigation of subsistence exposed to thermal neutron fluxes corresponding to those neutron fluxes expected from fission weapons. The investigations encompass correlation of experimental results with calculated values and the development of analytical methodology to continue the investigation. Since the last report, 92 samples have been exposed to nuclear radiation at four flux levels in the WRAIR reactor. A total of 162 samples have been exposed to five integrated fluxes since July 1963. These include three subsistence items and corresponding standards of sulfur and phosphorus. Methodology has been developed for accurate appraisal of P32 using the liquid scintillation system of counting for greatest efficiency, particularly for P32 activity at low flux neutron radiation. Results of P32 studies show good correlation to those values expected by mathematical calculation.		
9. KEY WORDS Neutron-induced radioactivity, Phosphorus 32, Sulfur 35, subsistence.		
10. SUPPORTING PROJECTS Not Applicable		
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet	12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet	
<div style="display: flex; justify-content: space-between;"> DA FORM 1309R 1 June 60 PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of 1 </div>		

ACCESSION NO.

36199

ARMY RESEARCH TASK REPORT

13. PROJECT, TASK OR
SUBTASK NUMBER

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
3	A	0	1	3	0	0	1	A	8	1	4	0	1	0	4			

14. DATE OF REPORT (30-33)

30 33 34

15. SECURITY OF WORK (34)

0 6 6 4 4

16. TYPE OF REPORT

35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53
1																		

17. SCIENTIFIC FIELD
a. Topical Classific. (56-61)
b. Functional Class (62-64)

56	57	58	59	60	61	62	63	64
0	1	0	4	0	0			

18. OSD CLASSIFICATION
(65-66)65 66
B R

19. R&D CATEGORY (67)

67
1

20. CONTRACT NUMBER

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
D	A															

21. GRANT NUMBER

28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46
D	A												G					

22. ESTIMATED COMPLET.
DATES

47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71
1	C	O	N	T	2					3					4					5				

23. PRIORITY (11-14)

11 14
124. PROGRAM ELEMENT
(15-26)15 26
6 • 1 1 • 3 0 • 0 1 • 1

25. CMR&D CODES

27	28	29	30	31	32	33	34	35
N	/	A						

26. CDOG REFERENCE

a. Paragraph No. (36-44)

b. Functional Group (45)

36	37	38	39	40	41	42	43	44	45
									6

27. FUNDING

a. Est. Total Cost (11-15)

b. % Spent Intern. (16-18)

" " Extern. (19-21)

c. Total Obligation (22-26)

d. Progmd. Cur. FY (27-33)

e. " " " +1 (34-40)

f. " " " +2 (41-47)

g. " " " +3 (48-54)

h. " " " +4 (55-61)

i. " " " +5 (62-68)

j. " " " +6 (69-75)

k. Total Man Years of

Effort (76-78)

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
					1			2							
27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42
43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58
59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74
75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90

DA FORM 1309R
1 June 63

Previous Editions are Obsolete

Page 2 of _____

ACCESSION NUMBER

36199

ARMY RESEARCH TASK REPORT*Continuation Sheet***PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:**

(A) Ellis, M. K., B. S., Dept of Food Radiomucclides
Div of Vet Med, WRAIR, WRAMC, Washington, D. C., 20012
576-3646 or Interdepartmental Code 198, Ext 3646 49

(A) Liddle, C. G., Capt, VC, Dept of Medicinal Chemistry
WRAIR, WRAMC, Washington, D. C., 20012
576-3568 or Interdepartmental Code 198, Ext 3568 49

REPORTS. Annual Progress Report, Walter Reed Army Institute of
Research, 1 July 1963 - 30 June 1964.

ANNUAL PROGRESS REPORT

Project No. 3A013001A814

Title: IN-HOUSE LABORATORY INITIATED R&D

Task No. 01

Title: In-House Lab Initiated R&D

Subtask No. 04

Title: Neutron-induced radioactivity in subsistence

Description:

The problem of neutron-induced activity by high thermal neutron fluxes is being studied in order to provide information for establishing guidelines for the handling of military subsistence items exposed to low yield nuclear weapons. Studies are being carried out to establish the levels of induced radioactivity to be expected, and to devise and standardize methods for detecting and quantitating levels of the more important radionuclides.

Progress:

1. A total of 162 samples, which include three subsistence items (tuna fish, dried eggs and canned ham) and standards of phosphorus and sulfur, have been exposed to different neutron fluxes (see sub para 2) employing the Walter Reed Army Institute of Research reactor. The subsistence items were analyzed for stable phosphorus by accepted Association of Official Agricultural Chemists methods in order to determine the quantity of phosphorus originally present.

2. In preliminary studies, all three subsistence items were used. Since the results from all three items were comparable at one flux level, attention was focused on samples of canned ham. They were exposed to six integrated flux levels ranging from 7×10^{10} to 1.6×10^{14} neutrons/cm². The range of neutron exposure was selected to bracket the approximate exposure that might result from the instantaneous dose delivered by a nuclear weapon. All samples were exposed in the reactor thermal column. There were six ham samples and six standards of ammonium phosphate ($\text{NH}_4\text{H}_2\text{PO}_4$) containing the equivalent phosphorus of the ham samples at each flux level. Each ham sample was exposed along side a standard in the reactor.

3. After removal from the reactor, the samples were surveyed for gamma activity using a 400 channel analyzer. Activated sodium as sodium²⁴ was detected and will be one of the elements to be investigated in more detail in future studies.

4. The samples were ashed, then extracted by the butyl alcohol solvent extraction method for phosphorus as reported in DASA 40-61, Subtask 03.038, 1963, to remove phosphorus³² from any other induced activities present. The stable phosphorus content served as the carrier for the radioactive phosphorus. Four gram samples were used for tuna fish and canned ham samples for activation, one gram samples were used in the dried egg samples.

5. The counting methods were of two types: (1) the GM gas-flow system with an efficiency of 17% and (2) the liquid scintillation system with an efficiency of 80% for phosphorus³². In the GM gas-flow system, the solvent was placed in planchets. Excessive self-absorption and creeping of the sample on the planchets made the liquid scintillation system more practical. The method employed for the liquid scintillation system was developed in this laboratory. The solvent containing the phosphorus was placed in a liquid scintillation counting vial, and to this was added absolute ethyl alcohol, hyamine hydroxide in methanol, and toluene containing PPO and POPOP. Hyamine hydroxide completely destroyed the color and no precipitation occurred. Since there was no loss of efficiency due to color or chemical quenching, it was not necessary to count using the channels ratio method. A recovery factor including efficiency for all samples ranged from 56 to 58%.

6. The activated standards were used to measure the exact neutron flux of the reactor employing the equation:

$$A = \phi \sigma N \sum_i P_i (1 - e^{-\lambda_i t}) + P_2 (1 - e^{-\lambda_2 t}) \dots P_i (1 - e^{-\lambda_i t})$$

The flux measurement was used to determine the expected empirical activity produced and then compared to the experimental results. For this calculation, the activation equation was used, i.e.

$$A = \phi \sigma N (1 - e^{-\lambda t}) e^{-\lambda t'}$$

The results are seen in the table that follows:

Empirical Activity (uc/4g)	Experimental Activity (uc/4g)	Standard Deviation	Flux (Neutrons per cm ²)
9.8 x 10 ⁻²	9.05 x 10 ⁻²	±1.2 x 10 ⁻²	1.64 x 10 ¹⁴
5.59 x 10 ⁻²	4.7 x 10 ⁻²	±0.6 x 10 ⁻²	9.4 x 10 ¹³
2.77 x 10 ⁻²	2.08 x 10 ⁻²	±0.29 x 10 ⁻²	4.62 x 10 ¹³
6.58 x 10 ⁻³	6.94 x 10 ⁻³	±0.91 x 10 ⁻³	1.27 x 10 ¹³
5.28 x 10 ⁻⁴	5.52 x 10 ⁻⁴	±0.8 x 10 ⁻⁴	7.85 x 10 ¹¹
5.1 x 10 ⁻⁵	5.12 x 10 ⁻⁵	±0.8 x 10 ⁻⁵	7.0 x 10 ¹⁰

It will be noted that the calculated activity falls within two standard deviations of all experimental values.

7. The half-life and beta energy curves after extraction of phosphorus³² were studied and were in agreement with the accepted values of 14.3 days and 1.71 MEV maximum beta energy, respectively.

8. Activated ham samples were also sent to the Army area laboratories for radiochemical analysis. The results indicate that an ability for determination of activity and for radiochemical separation exists in the participating laboratories. Further collaboration with the Army area laboratories is needed to continue their development and maintenance of radiochemical proficiency.

Summary and Conclusions:

1. Laboratory analyses for activated phosphorus are reproducible and constant within the errors of measurement. Over wide flux ranges, the activity of neutron-induced phosphorus³² is similar to values expected from the calculation using standard activation equations. The use of the nuclear reactor to simulate weapons effects for neutron activation seems practical. The half-life and beta energy values obtained after solvent extraction of the phosphorus agree with those of phosphorus³² such that the method of extraction eliminates all other induced activities.

2. Evaluation studies reveal that the U. S. Army medical laboratories possess the capability for radiochemical analysis of phosphorus³².

3. Studies are being continued.

ARMY RESEARCH TASK REPORT		REPORTS CONTROL SYMBOL CSCRD-6(R2)
ACCESSION NUMBER 36200		PROJECT, TASK, OR SUBTASK NO. 3A013001A81140105
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D. C., 20315	2. FUNDING AGENCY Army Medical R&D Command Office of The Surgeon General Washington, D. C., 20315	
3. CONTRACTING AGENCY NA	4. CONTRACTOR AND/OR GOV'T LABORATORY A Walter Reed Army Inst of Resch Walter Reed Army Medical Center Washington, D. C., 20012 723-1000, Ext 3552	
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Crosby, W. H., Colonel, MC, Dept of Hematology Div of Medicine, WRAIR, WRAMC, Washington, D. C., 20012 576-3365 or Interdepartmental Code 198, Ext 3365 *See Continuation Sheet		
6. TITLE OF: PROJECT <input type="checkbox"/> TASK <input type="checkbox"/> SUBTASK <input checked="" type="checkbox"/> Plasma volume expanders in relation to bleeding (U)		
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964		
8. RESUME (U) Independent investigations of mild bleeding disorders and of new dextran products merged when it was found that intravenous infusions of dextran produced a transient platelet factor 3 deficiency (thrombocytopenia). Platelet factor 3 has been assayed in 125 patients with various disorders in which a bleeding diathesis may occur. It was found to be abnormal in a significant percentage of patients with multiple myeloma, hyperglobulinemia, etc., diseases in which macromolecules are present in the serum. Intravenous infusions of standard dextran and low molecular weight dextran produced a transient marked deficiency of platelet factor 3 activity in 95 per cent of normal subjects tested. The platelet defect was correctable by sonic oscillation indicating that the platelets were not intrinsically deficient in factor 3, but that dextran coating prevented its release. The present investigations have demonstrated that failure of the dextran-coated platelets to release factor 3 contributes to the hemostatic defect due to dextran. The secondary thrombocytopenia due to dextran was identical to that observed in patients with various disease states. Corticosteroids have been found to protect platelets against the dextran induced platelet factor 3 deficiency <u>in vitro</u> but preliminary <u>in vivo</u> studies have been unsuccessful.		
9. KEY WORDS Bleeding, dextran, platelets, hyperglobulinemia, plasma, blood volume expanders, corticosteroids.		
10. SUPPORTING PROJECTS Not Applicable		
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet	12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet	
DA FORM 1309R 1 June 63		PAGE 1 of 1

ACCESSION NO.

36200

ARMY RESEARCH TASK REPORT

13. PROJECT, TASK OR
SUBTASK NUMBER

11	22	23 24	25 26	27 28	29
3 A 0 1 3 0 0 1 A 8 1 4	0 1	0 5			

14. DATE OF REPORT (30-33)

30	33	34
0 6 6 4	4	

15. SECURITY OF WORK (34)

16. TYPE OF REPORT

35	36	47 48 49 50 51	52	53
1				

17. SCIENTIFIC FIELD

 a. Topical Classific. (56-61)
 b. Functional Class (62-64)

56	61	62	64
0 1 0 6 0 3			

18. OSD CLASSIFICATION

(65-66)

19. R&D CATEGORY (67)

65 66	67
B R	1

20. CONTRACT NUMBER

11 12	13 14	15	17	18	21	22	26	27
D A								

21. GRANT NUMBER

28 29	30	33	34 35	36	38	39 40	41	45	46
D A							G		

22. ESTIMATED COMPLET.
DATES

47	51	52	56	57	61	62	66	67	71
1 C O N T	2			3		4		5	

23. PRIORITY (11-14)

24. PROGRAM ELEMENT
(15-26)

11	14	15	26
	1	6 • 1 1 • 3 0 • 0 1 • 1	

25. CMR&D CODES

27	29	30	32	33	35
N / A					

26. CDOG REFERENCE

a. Paragraph No. (36-44)

b. Functional Group (45)

36	39	40	41 42	43 44	45
					6

27. FUNDING

a. Est. Total Cost (11-15)

b. % Spent Intern. (16-18)

" " Extern. (19-21)

c. Total Obligation (22-26)

d. Program. Cur. FY (27-33)

e. " " " +1 (34-40)

f. " " " +2 (41-47)

g. " " " +3 (48-54)

h. " " " +4 (55-61)

i. " " " +5 (62-68)

j. " " " +6 (69-75)

k. Total Man Years of
Effort (76-78)

11	15	16	18	19	21	22	26
		1		2			
27 28	29	33	34 35	36	40		
41 42	43	47	48 49	50	54		
55 56	57	61	62 63	64	68		
69 70	71	75	76	78			

DA FORM 1309R
1 June 63

Previous Editions are Obsolete

Page 2 of _____

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

- (A) Ewald, R. A., Capt, MC, Dept of Hematology
Div of Medicine, WRAIR, WRAMC, Washington, D. C., 20012
576-3040 or Interdepartmental Code 198, Ext 3040 49
- (A) Sears, D. A., Capt, MC, Dept of Hematology
Div of Medicine, WRAIR, WRAMC, Washington, D. C., 20012
576-3040 or Interdepartmental Code 198, Ext 3040 49
- (A) Weiss, H. J., M.D., Dept of Medicine
New York University School of Medicine, New York, N. Y.
Area Code 212, OR 9-3200, Ext 2855 30
- (A) Eichelberger, J. W., B.S., M.T., Dept of Hematology
Div of Medicine, WRAIR, WRAMC, Washington, D. C., 20012
576-3385 or Interdepartmental Code 198, Ext 3385 49

REPORTS. Annual Progress Report, Walter Reed Army Institute of Research, 1 July 1963 - 30 June 1964.

Ewald, R. A. and Crosby, W. H.: Reported Experiences with Dextran in Communist Countries. Transfusion 3: 376, 1963.

Ewald, R. A., Anderson, P., Williams, H. L. and Crosby, W. H.: Effects of Intravenous Infusions of Feather Keratin: Preliminary Characterisation and Evaluation as a Plasma Expander. Proc. Soc. Exper. Biol. Med. 115: 130-133, 1964.

Weiss, H. J. and Eichelberger, Jr., J. W.: Secondary Thrombocytopathia. Arch. Int. Med. 112: 827-834, 1963.

ANNUAL PROGRESS REPORT

Project No. 3A01300LA814

Title: IN-HOUSE LABORATORY
INITIATED R&D

Task No. 01

Title: In-House Lab Initiated
R&D

Subtask No. 05

Title: Plasma volume expanders
in relation to bleeding

Description:

Independent investigations in this laboratory of mild bleeding disorders and of new dextran products merged when it was discovered that intravenous infusions of dextran produce a transient platelet factor 3 deficiency (thrombocytopathia). Previously, eight patients with undiagnosed bleeding disorders were found to have a platelet factor 3 deficiency as determined by a sensitive quantitative assay. Thus the development of a new method for detecting mild bleeding disorders and the means of producing the platelet abnormality in normal subjects offer a potential breakthrough in the study of coagulation defects. It is possible that the platelet dysfunction may explain the abnormal bleeding observed after large intravenous infusions of dextran.

Progress:

Thrombocytopathia has been found to occur as a primary disorder (idiopathic) or in the presence of underlying disease (secondary). In addition, family studies have revealed that the abnormality of platelet factor 3 activity may be inherited as a Mendelian dominant.

In the majority of cases of thrombocytopathia the defect was found to be due to an abnormal serum factor which interfered with platelet factor 3 release. Thus incubation of the patient's serum with normal platelets produced a platelet factor 3 deficiency in those platelets. Also disruption of abnormal platelets with sonic oscillation resulted in markedly increased factor 3 activity, indicating that the basic defect was inability of the platelets to release factor 3, rather than an absolute deficiency of it.

Platelet factor 3 has been assayed in 125 patients with various disorders in which a bleeding diathesis may occur. It was found to be abnormal in a significant percentage of patients with multiple myeloma, hyperglobulinemia, etc., diseases in which macromolecules are present in the serum. The similarity of these disorders to the presence of dextran molecules in the serum following infusion is apparent. In the cases studied there was a reasonably good correlation between abnormal platelet function and clinical bleeding.

Administration of corticosteroids has temporarily corrected the platelet abnormality in a number of patients, and has permitted operative procedures to be carried out in these patients without abnormal bleeding.

A striking feature in patients with idiopathic thrombocytopathia is the high incidence of allergy or other evidence of hypersensitivity in the family history. There is some evidence to suggest that the platelet abnormality is related to an abnormal immune response.

The hemostatic defect (prolongation of the bleeding time) due to standard dextran infusions was first reported from this laboratory in 1954. The mechanism of the dextran induced defect was not known, but further studies indicated that dextran in some way interfered with normal platelet function. The present investigations have demonstrated that failure of the dextran-coated platelets to release factor 3 contributes to the hemostatic defect.

Intravenous infusions of standard dextran and low molecular weight dextran produced a transient marked deficiency of platelet factor 3 activity in 95 per cent of the normal subjects tested. Infusions of 5 per cent albumin solutions had no effect on platelet function. The decrease in factor 3 activity was related to both the concentration and molecular weight of the infused dextran. The platelet defect was correctable by sonic oscillation indicating that the platelets were not intrinsically deficient in factor 3, but that dextran coating prevented its release. These findings were confirmed by in vitro incubation studies. In 5 of 7 subjects who developed prolongation of the bleeding time and/or positive tourniquet tests, there was a direct relationship to the lowest platelet factor 3 activity. There was no direct relationship of factor 3 deficiency to hemodilution, total platelet counts or platelet adhesiveness. A reasonably good correlation between dextran levels and depressed platelet factor 3 activity was observed.

The secondary thrombocytopathia due to dextran was identical to that observed in patients with various disease states. Corticosteroids have been found to protect platelets against the dextran induced platelet factor 3 activity in vitro, but preliminary studies in vivo have been unsuccessful.

A distinct relationship has been found to exist between the in vitro and in vivo sensitivity of platelets to dextran. The platelets of patients that are not rendered abnormal by intravenous infusions of dextran are resistant in vitro, also. This relationship was further supported by studies in five subjects who had showed a significant factor 3 defect following dextran infusion. Incubation of their platelets with dextran resulted in a marked decrease of platelet factor 3 activity.

Normal platelets have shown a marked depression of factor 3 activity when incubated with serum from a subject who has been infused with dextran.

Summary and Conclusions:

Platelet factor 3 has been assayed in various disease states in which a bleeding diathesis may occur, and has found to be abnormal in a significant percentage of patients with multiple myeloma, hyperglobulinemia, etc., diseases in which macromolecules are present in the serum. Similarly, intravenous infusions of standard dextran and low molecular weight dextran produced a transient marked deficiency of platelet factor 3 activity in 95 per cent of normal subjects tested. The platelet defect was correctable by sonic oscillation indicating that dextran coating of platelets prevented release of factor 3. The secondary thrombocytopathia (platelet factor 3 deficiency) due to dextran was identical to that observed in patients with various disease states. These investigations have demonstrated that failure of dextran-coated platelets to release factor 3 contributes to the hemostatic defect due to dextran.

ARMY RESEARCH TASK REPORT				REPORTS CONTROL SYMBOL CSCRD-4(R2)	
ACCESSION NUMBER 36201			PROJECT, TASK, OR SUBTASK NO. 3A013001A8140106		
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D. C. , 20315			2. FUNDING AGENCY Army Medical R&D Command Office of The Surgeon General Washington, D. C. , 20315		
3. CONTRACTING AGENCY Hq, US Army Medical R&D Command Office of The Surgeon General Washington, D. C. , 20315			4. CONTRACTOR AND/OR GOV'T LABORATORY 4 University of Maryland College Park, Maryland 927-3800, Ext. 536		
18					
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Rollinson, Carl L. , PhD, Professor, Dept Chemistry University of Maryland, College Park, Maryland 927-3800, Ext. 536					
See Continuation Sheet 18					
6. TITLE OF: PROJECT <input type="checkbox"/> TASK <input type="checkbox"/> SUBTASK <input checked="" type="checkbox"/> Chromium complexes of insulin and related compounds(U)					
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964					
8. RESUME (U) A number of chromium compounds have been subjected to in vitro tests to determine the relation between their structure and their effectiveness in enhancing the action of insulin. The results indicate that the effect is strongly dependent on the composition of the chromium compound and in particular that the most active compounds are polynucleate, the polynucleate structure of those tested being due to olation. Investigation of synthetic methods for preparing such compounds of known chain length has been started. Preliminary work has been done on the possible formation of chromium complexes under conditions similar to those of the digestive system. Rapid methods for determining chromium have been studied; a spectrophotometric procedure described in the literature has been checked and is in use, and a method based on separation of Cr(OH) ₃ from organic matter is under investigation.					
9. KEY WORDS Chromium, insulin, chelate compounds, olation					
10. SUPPORTING PROJECTS Not applicable.					
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet			12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet		
<div style="display: flex; justify-content: space-between;"> <div>DA FORM 1309R 1 June 63</div> <div>PREVIOUS EDITIONS ARE OBSOLETE</div> <div>PAGE 1 of _____</div> </div>					

ACCESSION NO.

36201

ARMY RESEARCH TASK REPORT

13. PROJECT, TASK OR
SUBTASK NUMBER

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
3	A	0	1	3	0	0	1	A	8	1	4	0	1	0	6			

14. DATE OF REPORT (30-33)

30	31	32	33	34
0	6	6	4	4

15. SECURITY OF WORK (34)

16. TYPE OF REPORT

35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55
1																				

17. SCIENTIFIC FIELD

 a. Topical Classific. (56-61)
 b. Functional Class (62-64)

56	57	58	59	60	61	62	63	64
0	1	0	1	0	0			

18. OSD CLASSIFICATION

(65-66)

19. R&D CATEGORY (67)

65	66	67
A	R	R

20. CONTRACT NUMBER

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
D	A	4	9	1	9	3			M	D	0	2	4	4	4	

21. GRANT NUMBER

28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46
D	A												G					

22. ESTIMATED COMPLET.
DATES

47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71
1	0	5	6	6	2					3					4					5				

23. PRIORITY (11-14)

24. PROGRAM ELEMENT
(15-26)

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
			1	6	•	1	1	•	3	0	•	0	1	•	1

25. CMR&D CODES

27	28	29	30	31	32	33	34	35
N	/	A						

26. CDOG REFERENCE

a. Paragraph No. (36-44)

b. Functional Group (45)

36	37	38	39	40	41	42	43	44	45
									6

27. FUNDING

a. Est. Total Cost (11-15)

b. % Spent Intern. (16-18)

" " Extern. (19-21)

c. Total Obligation (22-26)

d. Progmd. Cur. FY (27-33)

e. " " " +1 (34-40)

f. " " " +2 (41-47)

g. " " " +3 (48-54)

h. " " " +4 (55-61)

i. " " " +5 (62-68)

j. " " " +6 (69-75)

k. Total Man Years of

Effort (76-78)

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
			3	0	1			2	0	0				3	0

27	28	29	30	31	32	33	34	35	36	37	38	39	40
6	4						6	5					

41	42	43	44	45	46	47	48	49	50	51	52	53	54
6	6						6	7					

55	56	57	58	59	60	61	62	63	64	65	66	67	68
6	8						6	9					

69	70	71	72	73	74	75	76	77	78
7	0							1	

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

(A) Mertz, W., M. D., Division Biochemistry,
WRAIR, WRAMC, Washington, D. C. 20012
576-3527 or Interdepartmental Code 198, Ext. 3527 49

(A) Lindsay, J. V., B. S., Dept Chemistry,
University of Maryland, College Park, Md.
927-3800, Ext. 536. 18

REPORTS. Annual Progress Report, Walter Reed Army
Institute of Research, 1 July 1963 - 30 June 1964.

ANNUAL PROGRESS REPORT

Project No. 3A013001A814 Title: In-House Laboratory Initiated R&D

Task No. 01 Title: In-House Lab Initiated R&D

Subtask No. 06 Title: Chromium complexes of insulin and related compounds.

Description: Chromium (III) has been established as a micro-nutrient essential for maintenance of normal glucose tolerance in the rat. Biological activity of various complexes was found to vary widely. The intent of this project is to correlate chemical structure with bioactivity, and, by correlating changes in bioactivity with well defined chemical alterations, to arrive at an understanding of the action of chromium (III) at the molecular level.

Progress: This investigation has progressed at a slower rate than was originally envisioned because of the unanticipated difficulty of obtaining qualified research assistants. Various efforts that have been made will probably solve this problem with the consequence that progress during the remainder of the three year period can be accelerated.

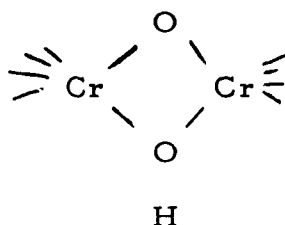
Since nothing was known about requirements for specific chemical structure in the biological activity of chromium, most of our effort has been devoted to exploratory experimental work and reviews of the pertinent literature. As a result of these activities, the course of the investigation for the immediate future appears to be clearly defined. Four quite specific but inter-related aspects are involved, namely: preparation, characterization and evaluation of chromium-insulin complexes; continuation of preparation and testing of a variety of chromium complexes; preparation of related chromium compounds of known composition; investigation of formation of chromium complexes from ligands possibly formed in the digestive process.

Chromium-Insulin Complexes - The macroscopic procedures ordinarily used in synthetic work would be useless for our purposes here because of the unavailability of insulin in large quantities and the high weight ratio of insulin to chromium in any expected complex, i. e. , a weight ratio of about 6000/52 in a 1:1 complex.

With the quantities of insulin that are readily obtained experiments can be performed involving 60 mg of insulin and 0. 52 mg of chromium. Separation of products from reaction mixtures can be accomplished by, for example, thin-layer chromatography; paper chromatography; electrophoresis; gel filtration; ion-exchange. The work to date on this part of the problem has been limited to acquiring information about the methods mentioned from the literature, apparatus suppliers and people who are using some of these techniques.

Preparation and Evaluation of Chromium Complexes - A considerable number of chromium (III) complexes have been prepared and submitted to WRAIR for testing of their ability to enhance the action of insulin in vitro. These include hexa-urea complexes, a-amino acid complexes, etc.

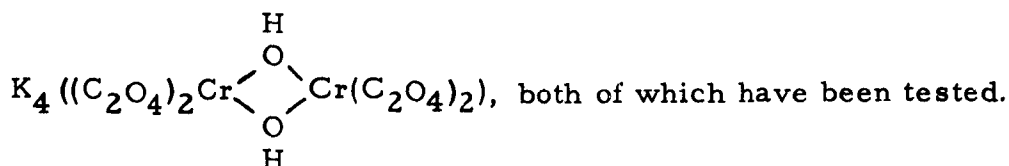
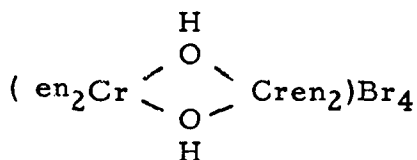
The results appear to warrant the conclusion that one requirement that the compound must satisfy is that it contain more than one chromium atom. With the compounds tested, the reaction responsible for formation of such compounds is olation, i. e. , chaining out because of bridging hydroxy groups.



Although other negative ions besides hydroxy can bridge, olation is such a common phenomenon in aqueous solutions that it warrants detailed investigation.

Olated Chromium Complexes - Most of the transition elements that form numerous coordination compounds undergo olation, but this reaction is especially characteristic of chromium (III). Since it results in liberation of hydrogen ions, it is promoted by bases; it is also accelerated by heat. If base is continually added to a Cr(III) solution, larger and larger olated aggregates are formed; the colloidal stage is reached and the final stage of the process is precipitation of chromium(III) hydroxide, which is biologically inert.

Some well-characterized olated chromium compounds of two and three chromium atoms have been reported (e. g.



However, the general problem, i. e., the deliberate preparation of compounds Cr_x where x is known to be 3, 4, 5 ---, has not been solved.

Almost all the work hitherto reported has been done with aqueous solutions. Under these conditions the water is not just a reaction medium; it is actually a 55.6 molar reagent solution, since every water molecule is a potential OH^- ion and can contribute to olation.

The indicated approach to the problem is therefore to utilize nonaqueous solvents and to provide only the concentration of OH^- to give the desired $\text{OH}^-/\text{Cr}^{+3}$ ratio. Preliminary work along these lines has been done; the solvents are N,N-disubstituted amides which are excellent solvents for many organic and inorganic substances.

Since reactions do not necessarily occur according to the equations one writes, a choice of methods for characterizing the reaction mixtures must be made. When a solid product is obtained, the usual analytical methods are usually sufficient. Indirect methods must be used if everything remains in solution, and it has been decided in the contemplated investigation to utilize primarily spectrophotometry and determination of conductivity (in addition to the empirical evaluation of reaction mixtures by the usual biological testing procedure).

Since the solvents of interest are somewhat hygroscopic, precautions are necessary to insure that the reaction mixtures remain dry during the various manipulations up to the point they can be put in sealed containers. A glove-box has been constructed for these manipulations, and some experimental work on drying the solvents has been done. Azeotropic dehydration with benzene, followed by fractionation, appears to be the most convenient procedure, and will be used if amides purified in this way have the conductance reported in the literature. Conductivity equipment is being assembled.

The ultimate objective is to obtain polynucleate chromium complexes containing known numbers of chromium atoms and having deliberately chosen end-groups.

Formation of Chromium Complexes in the Digestive Process - The results obtained by in vitro tests show that the effectiveness of chromium(III) on the action of insulin is strongly dependent on the composition of the compound. On the other hand, it has been found that chromium present in the food of experimental animals seems to be effective almost irrespective of the compound, e. g., even chromium chloride and chromium sulfate may be used.

This leads to the conclusion that chromium(III) can be converted in the digestive system to effective chelates. Preliminary work designed to determine whether the relatively mild conditions of the living organism can convert chromium(III) to chromium complexes has been performed.

The synthetic chemist, in attempting to make a compound, is inclined to boil the reaction mixture, use extremely high concentrations of reagents, and is usually in a hurry. In the experiments mentioned, the reaction mixture was kept at 37° and mixing of reagents of (quite low concentration) was carried out over a period of 2-3 hours. The idea was to simulate the process in which acid contents of the stomach become neutralized in the intestine.

It may be imagined that, as the contents of the stomach enter the intestines, and the pH is raised, competitive and consecutive reactions involving chromium will occur, i. e., olation and chelation. If the first is predominant, it would be possible for it to go so far as to give high molecular weight products incapable of diffusing through the intestine wall, or even chromium hydroxide. This may account for the fact that only a small proportion of orally administered $\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$ is actually absorbed.

At any rate, in experiments in which the pH of a chromium(III) chloride amino acid solution at 37°C was slowly raised with Na_2CO_3 , no precipitation occurred, but rather, the green solution gradually turned red-violet. Some hours after conclusion of the heating, violet precipitates formed. The products, it is believed, are amino-acid olated chromium chelates; analytical work to characterize the solids is still in progress.

Chemical Analysis of Chromium Compounds -

In synthetic work, one of the time-consuming activities is analysis of products. In the work under discussion this always requires determination of chromium and generally halide. Whenever a chromium complex with an organic ligand is of sufficient interest, microanalysis for C, H, N and sometimes S is indicated.

For the day-to-day work, chromium determination is essential. A method that is satisfactory in accuracy and precision comprises destruction of organic matter with perchloric acid followed by neutralization and addition of sodium peroxide to insure complete oxidation of chromium to Cr^{+6} . It is, however, somewhat slow, and there is always a potential hazard in the use of perchloric acid.

Almost all compounds of interest in this work contain organic ligands and quantitative oxidation of chromium is impossible unless the organic matter is destroyed or removed. As an alternative to oxidizing it with perchloric acid, separating it from the chromium is a promising possibility.

Chromium is precipitated as the hydroxide. Filtration is entirely too slow, so the procedure adapted is to precipitate the hydroxide in a centrifuge tube, spin it down, suspend it in the same tube in the wash solution, centrifuge, etc. The resulting $\text{Cr}(\text{OH})_3$ can then be oxidized by any of the standard oxidants, and halogen can be determined in the combined supernatant and wash solutions.

The perchloric acid procedure gives results of the accuracy and precision usually expected of good oxidimetric procedures. It is anticipated that the alternative method described will be equally good in these respects.

A very fast method, even though it does not give as accurate results as the above, is also desirable for routine use. A spectrophotometric method described by Theis and Serfass has been checked and has been found extremely useful for quickly determining the chromium content of a compound within $\pm 2\%$.

The complex, dissolved or suspended in water is heated with an excess of oxalic acid. The oxalate ion is such a strong ligand that it can convert any chromium complex to $(Cr(C_2O_4)_3)^{-3}$. The % transmittance of the solution (about .01-.02 M) is determined at 490 mu and the chromium concentration is read off a chart of % transmittance vs. concentration of solutions of known chromium content.

Summary & Conclusions: It was shown that the in vitro biological activity of chromium compounds depends on the state ofolation as well on the nature of ligands.

Mononuclear as well as polynuclear complexes of high molecular weight are biologically ineffective. Bioactivity increases initially with the degree of olation. The definition of optimal size is being attempted.

Preliminary experiments, still in progress, suggest that certain amino acids as ligands result in compounds of greater activity than all previously tested. This fact is being investigated with regard to the formation of chromium complexes from dietary ingredients in the gastrointestinal tract.

ARMY RESEARCH TASK REPORT				REPORT CONTROL SLIP CSCRD-5(R2)
ACCESSION NUMBER 36202			PROJECT, TASK, OR SUBTASK NO. 3A013001A8140107	
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D. C. 20315		2. FUNDING AGENCY Army Medical R&D Command Office of The Surgeon General Washington, D. C. 20315		
3. CONTRACTING AGENCY HQ, US Army Medical R&D Command Office of The Surgeon General Washington, D. C. 20315		4. CONTRACTOR AND/OR GOV'T LABORATORY 4 Georgetown University 1834 Connecticut Avenue Washington, D. C. 333-2000		
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Geschickter, C. F., M. D. Georgetown University, 1834 Connecticut Avenue, Washington, D. C. 333-2000, Ext 821 See Continuation Sheet				
6. TITLE OF: PROJECT <input type="checkbox"/> TASK <input type="checkbox"/> SUBTASK <input checked="" type="checkbox"/> Intrapulmonary infusion of fluids (U)				
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964				
8. RESUME (U) Preliminary work on this contract led to an extension of the research aim into the changes in the microcirculation which occur in various forms of shock from the earliest to the latest reversible stages. The time sequence, localization and character of the microcirculatory changes in shock as demonstrated by histopathology and vital dye techniques have been examined with particular reference to the role of hemolysis and intravascular coagula in lower animals and in man. As the composition of the intravascular coagula was unknown special histologic techniques were applied to identify the component parts. The chromic acid-Schiff stain (Gridley), Lassaramin Fast Red, and the phosphotungstic acid hematoxylin-acid eosin stain were particularly useful in demonstrating hemoglobin and various densities of fibrin deposition. Thirty-two human autopsy cases of death with a clinical diagnosis of profound shock were searched for evidence of any premortem intravascular clotting phenomena. These findings were compared with a control group. Similar studies were carried out in dogs, rabbits and monkeys. No evidence of disseminated vascular clotting was noted in the control series, while red cell and fibrin agglomerations were found in the shock groups. The use of immuno-fluorescent techniques and autoradiographic techniques is planned.				
9. KEY WORDS Morphology, microcirculation, shock, coagulation, fibrin, erythrocytes.				
10. SUPPORTING PROJECTS Not Applicable				
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet			12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet	
DA FORM 1309R 1 Jun 63			PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of _____	

ACCESSION NO.

36202

ARMY RESEARCH TASK REPORT

13. PROJECT, TASK OR
SUBTASK NUMBER

11	22	23 24	25 26	27 28	29
3 A 0 1 3 0 0 1 A 8 1 4		0 1	0 7		

14. DATE OF REPORT (30-33)

30	33	34
0 6 6 4		4

15. SECURITY OF WORK (34)

16. TYPE OF REPORT

35	36	47 48 49 50 51	52	53
1				

17. SCIENTIFIC FIELD

 a. Topical Classific. (56-61)
 b. Functional Class (62-64)

56	61	62	64
0 1 0 6 0 7			

18. OSD CLASSIFICATION

(65-66)

19. R&D CATEGORY (67)

65 66	67
B R	1

20. CONTRACT NUMBER

11 12	13 14	15 17	18	21	22	26	27
D A	4 9	1 9 3		M D	0 2 5 1 4		

21. GRANT NUMBER

28 29	30	33	34 35	36 38	39 40	41	45	46
D A						G		

22. ESTIMATED COMPLET.
DATES

47	51	52	56	57	61	62	66	67	71
1 0 9 6 4		2		3		4		5	

23. PRIORITY (11-14)

24. PROGRAM ELEMENT
(15-26)

11	14	15	26
	1	6 • 1 1 • 3 0 • 0 1 • 1	

25. CMR&D CODES

27	29	30	32	33	35
N / A					

26. CDOG REFERENCE

a. Paragraph No. (36-44)

b. Functional Group (45)

36	39	40	41 42	43 44	45
					6

27. FUNDING

a. Est. Total Cost (11-15)

b. % Spent Intern. (16-18)

" " Extern. (19-21)

c. Total Obligation (22-26)

d. Progmd. Cur. FY (27-33)

e. " " " +1 (34-40)

f. " " " +2 (41-47)

g. " " " +3 (48-54)

h. " " " +4 (55-61)

i. " " " +5 (62-68)

j. " " " +6 (69-75)

k. Total Man Years of

Effort

(76-78)

11	15	16 18	19 21	22	26
	4 2	1	2 0 0		4 2
27 28	29	33	34 35	36	40
6 4		4 2			
41 42	43	47	48 49	50	54
55 56	57	61	62 63	64	68
69 70	71	75	76	78	
				3	

ACCESSION NUMBER

36202

ARMY RESEARCH TASK REPORT**Continuation Sheet**

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

(A) Chun, Byungkyu, M.D., Div of Clin Surg, WRAIR,
WRAMC, Washington, D. C. 20012, and Dept of Path-
ology, Georgetown Univ Sch of Med, Washington, D.C.
576-3477 or Interdepartmental Code 198, Ext 3477 49

(A) Hardaway, Robert M., Col, MC, Div of Clin Surg,
WRAIR, WRAMC, Washington, D. C. 20012
576-3669 or Interdepartmental Code 198, Ext 3669 49

REPORTS. Annual Progress Report, Walter Reed Army Institute
of Research, 1 July 1963 - 30 June 1964.

ANNUAL PROGRESS REPORT

Project No. 3A013001A814

Title: In-House Laboratory
Initiated R&D

Task No. 01

Title: In-House Lab Initiated
R&D

Subtask No. 07

Title: Intrapulmonary infusion
of fluids

Description: Evidence of disseminated intravascular coagulation (DIC) in capillaries and other microvessels as the etiology of experimental hemorrhagic, endotoxin, traumatic, and other types of shock in dogs has recently been presented by Hardaway et al. of WRAIR. The present paper is the result of histologic study of organs of animals and humans subjected to various types of shock.

Progress:

Materials and Methods.

Human cases of shock. Forty-two clinical cases form the basis for this study. They were divided into two groups.

Group 1. Records on 32 cases of death with a clinical diagnosis of profound shock, regardless of the primary cause, were obtained from the registrars of the Walter Reed General Hospital and Georgetown University Medical Center. The causes of death on each case were clarified by a complete necropsy procedure at the respective medical centers. The clinical situations were carefully evaluated and microscopic slides were thoroughly reviewed with particular attention to any premortem intravascular clotting phenomena. The tissue changes seemingly related to intravascular clotting were recorded. In many instances, corresponding paraffin blocks were resectioned and stained with Hematoxylin and Eosin, Gridley E. histolytica stain, Lassaramin Fast Red, or PTAH with Acid Eosin. The last three stains were used for the specific purpose of investigating the relationship between the red cells or hemoglobin and the proteinacious material covering the red cells. Red cells as well as extruded hemoglobin stained rose red color with three stainings, specifically with Gridley method. Fibrin stained green with Gridley's, yellow with Lassaramin Fast Red and blue or dark blue with PTAH. The PTAH is considered to be the most valuable for well formed fibrin.

Group 2. Ten cases of death without a clinical diagnosis of shock were reviewed from records of Georgetown University Medical Center. Seven were stillbirths and three were adults who were killed instantly in automobile accidents. All had a complete necropsy and microscopic slides were reviewed as in Group 1.

Dogs.* Thirty-two dogs were bled to 50 mm. Hg. mean arterial pressure and kept at this level for 2½ hours by removing or restoring small amounts of blood. All blood was reinfused at the end of the shock period. Liver and lung biopsies were taken before hemorrhage, after 90 minutes of hypotension, and at the end of the shock period. Autopsies were done and sections made immediately on death which occurred within 24 hours in 75% of the animals.

Rabbits.* One-half ml. of *E. coli* endotoxin (prepared by Walter Reed Army Institute of Research Department of Bacteriology) was administered intravenously to five rabbits. The following day another ½ ml. dose of endotoxin was administered. All of the rabbits died within 3 hours. Rabbits were sacrificed just before death and tissue sections made.

Monkey.* A monkey dying of dysentery and probably septicemia caused by *Shigella* organisms was autopsied immediately at death and tissue sections taken.

Results.

Human cases.

Group 1. Eleven cases or 34.4% were proved to have septicemia with Gram negative pathogens such as *E. coli*, *Pseudomonas*, *Proteus*, Friedlander's bacillus, paracolon B, and *E. typhosa*. Other cases of gram negative septicemia were clinically suspected but unproven. In addition there were cases of gram positive sepsis such as *Streptococcus faecalis*.

Massive and continuous hemorrhage of various causes occurred in 13 or 40.6% of cases.

One or 3.1% of cases of shock resulted primarily from massive trauma. Three or 9.5% of cases had leukemia and three or 9.4% had other types of malignancy. Six or 18.8% had acute pancreatitis. Three or 9.4% had a complication of pregnancy.

In 16 cases some pertinent hematologic data (platelet count and prothrombin time) were recorded. The average platelet count of all cases in which it was available was 40,000. The average prothrombin was 61% of normal. Data on other blood coagulation factors, clotting times, etc., were not obtained. The available hematological data was compatible with that obtained in experimental shock in animals, and reflects a characteristic clotting defect. Clotting factors are depleted in an intravascular clotting episode.

*The principles of laboratory animal care as promulgated by the National Society for Medical Research were observed.

In 29 cases or 90.6%, there was evidence of intravascular clotting in various organs, most frequently in venules and glomerular tufts of kidneys, portal branches of liver, pancreatic venules, alveolar capillaries, capillaries in myocardium and cerebral vessels. One additional case showed sludged red cells. The most fascinating observation was the presence of rounded or ovoid sharply demarcated globules ranging in diameter from 10 - 100 or more micra which occasionally exhibited refractile appearance under the ordinary light microscope. Some globules showed centrally located bubble-like areas. Some smaller globules are covered with fine feather-like material arranging in radial fashion. Many times the globules are conglomerated into a large mass. The center of the smaller masses stained rose red color with Gridley's, Lassaramin Fast Red, and Acid Eosin indicating a red cell as the center. The outer coat stained blue with PTAH. The radial feather-like material around the mass also stained dark blue with PTAH. Probably the center mass is hemoglobin and the outer layer and feather-like material on the periphery is fibrin. The larger globules with bubble-like centers seemed to be totally composed of fibrin with trapped or destroyed red cells. The extruded hemoglobin is incorporated with the fibrin mass and is located in the periphery of the globular clots.

The mechanism of formation of these globules may be as follows: The surface of red cells under certain circumstances begins to accumulate fibrin. The red cells then start to degenerate. These fibrin coated red cells may be precursors of the globules. As the fibrin layer thickens, the cells may conglomerate and form a large globule. Or they may continue to circulate, adding layers of fibrin till a very large globule is formed. Under the proper circumstances the globules may begin to accumulate looser, radially arranged strands of fibrin and may stick to each other in an enlarging clot. The red cells within the globules gradually disintegrate and release their contents including hemoglobin which is mainly distributed in the periphery of the globule. (Red cell contents contain a clotting stimulant.) These globular clots circulate and eventually may occlude small sized vessels. They then may produce areas of coagulation necrosis in various organs. Before actual necrosis occurs, edema and inflammation may be seen. This is strong evidence for the premortem formation of these clots. In addition, clots of varying size are seen circulating with obviously normal red cells. When the clots are formed in the mucosal capillaries of the gastrointestinal tract, the surface may be sloughed thus producing gastrointestinal bleeding.

Group 2. No evidence of intravascular clotting and no globular clots were found in any Group 2 cases even after intensive study using experience gained in studying the Group 1 cases.

Dogs. Liver biopsies taken before hemorrhage consistently showed blood vessels to contain red cells well separated and not stuck together. At the end of the shock period there was Rouleaux formation and agglutination of red cells. If sections were stained with PTAH for fibrin, it was found that many but not all red cells take a fibrin stain. While this does not prove that the cells have a coat of fibrin it is compatible with it. Aggregates of red cells thus coated may occlude the microcirculation. At autopsy at death the occlusion was more compact and frequently found associated with focal tissue necrosis in the liver and kidneys.

Rabbits. After injection of E. coli endotoxin there were found in all of the rabbits rounded bodies of various sizes ranging from the size of red cell upward. These could occlude the microcirculation and were frequently found associated with focal necrosis in the liver and kidney. Fibrin thrombi were found in the lungs and other organs.

Monkey. Sections from the monkey dying in shock with Gram negative dysentery and probable septicemia showed globules in vessels and focal necrosis similar to those discussed above.

Discussion.

Shock may be defined as an inadequate capillary perfusion. Numerous factors may effect capillary perfusion. Arterial hypotension is one factor but another important one is arteriolar constriction. Usually these two phenomena occur together in shock and result in a greatly decreased flow through the arteriole. Cells which have previously been satisfied with perhaps 20% of time being perfused by their capillary, now require full time perfusion. Acting possibly through liberation of histamine by mast cells as discussed by Shayer, all capillaries now open. This combination of low arteriolar flow plus opening of all capillaries at one time cause extremely slow capillary flow. The flow is so slow that by the time the blood gets to the venous end of the capillary it is laden with lactic acid and is acidotic. The arteriovenous pH difference may increase from 7.4/7.3 to 7.4/6.9 within a few minutes. This acid pH has a great stimulating effect on blood coagulation. Other factors which may be present and also stimulate coagulation are hemolysis, endotoxemia, and high levels of blood clotting factors. The combination of extremely slow capillary flow plus the increased tendency of the blood to clot causes conversion of fibrinogen to fibrin and increased stickiness of platelets. The largest surface available for

deposition of fibrin is the surface of red cells. Deposition of a fibrin coat on red cells may account for their sticking together as in the formation of blood sludge, with occlusion of capillaries and other microvessels. This is reversible with endogenous or exogenous fibrinolysin, or by improving flow by volume replacement or by opening up the arterioles with a vasodilator. However, if conditions are allowed to continue untreated, and the capillaries are allowed to remain occluded for some period of time, cellular death may result. This may produce focal necrosis in vital organs such as the liver and kidney with death in liver or kidney failure (irreversible shock).

Summary and Conclusions:

Shock in humans, dogs, rabbits, and monkeys may produce a conversion of fibrinogen to fibrin. This may deposit on the largest surface available, namely, the red cells. This may cause a stickiness of the cells with agglutination. These cellular aggregates may coalesce into clots (DIC) which may occlude the microcirculation causing focal tissue necrosis in vital organs and death.

ARMY RESEARCH TASK REPORT		REPORTS CONTROL SYM CSCRD-6(R2)
ACCESSION NUMBER 58203		PROJECT, TASK, OR SUBTASK NO. 3A013001A8140108
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D. C., 20315	2. FUNDING AGENCY Army Medical R&D Command Office of The Surgeon General Washington, D. C., 20315	
3. CONTRACTING AGENCY NA	4. CONTRACTOR AND/OR GOV'T LABORATORY A Walter Reed Army Inst of Rsch Walter Reed Army Medical Center Washington, D. C., 20012 723-1000, Ext 3552	
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Williams, Harold L., Lt Col, MSC, Dept of Clinical and Social Psychology Division of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012 576-5257 or Interdepartmental Code 198, Ext 5257 See Continuation Sheet		
6. TITLE OF: PROJECT <input type="checkbox"/> TASK <input type="checkbox"/> SUBTASK <input checked="" type="checkbox"/> Effect of stress on tracking performance (U)		
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964		
8. RESUME (U) This is the final report on experiments using the Zero Input Tracking Analyzer (ZITA III). Projects proposed for FY-64 are completed. The tracking test was used in two studies of the effects of stress on human performance. The effect of sleep loss was to produce intermittent slowing, accompanied by loss of tracking control. Introduction of lag in the display tended to correct these errors. Distraction and information overload also produced gaps in control, but caused maximum decrement when the display contained lag. ZITA III is now incorporated into the battery of Walter Reed tests used in studies of the effects of sleep loss, fatigue and monotony on performance.		
9. KEY WORDS Tracking test, stress, decrement, sleep loss, human performance, information load, fatigue.		
10. SUPPORTING PROJECTS Not Applicable		
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet	12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet	
DA FORM 1309R 1 June 63 PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of		

ACCESSION NO.

36203

ARMY RESEARCH TASK REPORT

13. PROJECT, TASK OR
SUBTASK NUMBER

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
3	A	0	1	3	0	0	1	A	8	1	4	0	1	0	8			

14. DATE OF REPORT (30-33)

30	31	32	33	34
0	6	6	4	4

15. SECURITY OF WORK (34)

34
4

16. TYPE OF REPORT

35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55
2																	0	6	6	4

17. SCIENTIFIC FIELD

 a. Topical Classific. (56-61)
 b. Functional Class (62-64)

56	57	58	59	60	61	62	63	64
0	1	0	8	0	3			

18. OSD CLASSIFICATION
(65-66)

65	66	67
A	R	1

19. R&D CATEGORY (67)

20. CONTRACT NUMBER

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
D	A															

21. GRANT NUMBER

28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46
D	A												G					

22. ESTIMATED COMPLET.
DATES

47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71
1	1	2	6	3	2					3					4					5				

23. PRIORITY (11-14)

24. PROGRAM ELEMENT
(15-26)

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
			1	6	1	1	3	0	0	1	1				

25. CMR&D CODES

27	28	29	30	31	32	33	34	35
N	/	A						

26. CDOG REFERENCE

 a. Paragraph No. (36-44)
 b. Functional Group (45)

36	37	38	39	40	41	42	43	44	45
									6

27. FUNDING

a. Est. Total Cost (11-15)

b. % Spent Intern. (16-18)

" " Extern. (19-21)

c. Total Obligation (22-26)

d. Progrmd. Cur. FY (27-33)

e. " " " +1 (34-40)

f. " " " +2 (41-47)

g. " " " +3 (48-54)

h. " " " +4 (55-61)

i. " " " +5 (62-68)

j. " " " +6 (69-75)

k. Total Man Years of
Effort (76-78)

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
					1			2							
27	28	29	30	31	32	33	34	35	36	37	38	39	40		
41	42	43	44	45	46	47	48	49	50	51	52	53	54		
55	56	57	58	59	60	61	62	63	64	65	66	67	68		
69	70	71	72	73	74	75	76	77	78						

DA FORM 1309R
1 June 63

Previous Editions are Obsolete

Page 2 of _____

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

(A) Lubin, A., Ph.D., Dept of Clinical and Social Psychology,
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012
576-5251 or Interdepartmental Code 198, Ext 5251 49

(A) Giesecking, C. F., M.A., Dept of Clinical and Social Psychology,
Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012,
576-5251 or Interdepartmental Code 198, Ext 5251 49

REPORTS. Annual Progress Report, Walter Reed Army Institute of
Research, 1 July 1963 - 30 June 1964.

For previous year refer to Incl 11, Ltr, MEDEC-Z, HQ,
WRAIR, 1 July 1963, subject: In-House Laboratory
Director's Program, Reports Control Symbol CSCRD-
03(OT)-72, to Asst Secty of Army (R&D).

ANNUAL PROGRESS REPORT (FINAL)

Project No.	3A013001A814	Title: In-House Laboratory Initiated R&D
Task No.	01	Title: In-House Lab Initiated R&D
Subtask No.	08	Title: Effect of Stress on Tracking Performance

Description:

The intent of this investigation was to develop a Zero Input Tracking Analyzer to be used as part of a battery of performance measures in studies of stress. A successful instrument was developed by Norman K. Walker Associates and incorporated into Walter Reed performance measurement battery.

Summary and Conclusions:

Studies of the effect of sleep loss on tracking control showed that under sleep loss intermittent periods of slowing occurred. Introduction of lag tended to correct this behavior. On the other hand, introduction of lag in the display enhanced the deleterious effect of information overload.

ARMY RESEARCH TASK REPORT			REPORTS CONTROL SYMBOL CSCRD-4(R1)
ACCESSION NUMBER <div style="text-align: right; margin-right: 50px;">36204</div>		PROJECT, TASK, OR SUBTASK NO. <div style="text-align: right;">3A013001A8140109</div>	
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D. C., 20315	2. FUNDING AGENCY Army Medical R&D Command Office of The Surgeon General Washington, D. C., 20315		
3. CONTRACTING AGENCY <div style="text-align: center;">NA</div>	4. CONTRACTOR AND/OR GOV'T LABORATORY A Walter Reed Army Inst of Rsch Walter Reed Army Medical Center Washington, D. C., 20012 723-1000, Ext 3552 <div style="text-align: right; border: 1px solid black; width: 20px; float: right;">49</div>		
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Branche, W. C., Jr., M. S., Dept of Bacteriology, Div of Communicable Disease & Immunology, WRAIR, WRAMC, Washington, D. C., 20012 576-3758 or Interdepartmental Code 198, Ext 3758 See Continuation Sheet <div style="text-align: right; border: 1px solid black; width: 20px; float: right;">49</div>			
6. TITLE OF: PROJECT <input type="checkbox"/> <div style="margin-left: 40px;"> TASK <input type="checkbox"/> SUBTASK <input checked="" type="checkbox"/> Growth of <u>Entamoeba histolytica</u> in tissue cultures (U) </div>			
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964			
8. RESUME (U) <u>Entamoeba histolytica</u> has been grown in continuous monolayer tissue culture for a period of forty days. To date all bacterial contaminants have been eliminated with the exception of a single highly resistant strain of proteus. Present status: Completed.			
9. KEY WORDS <u>Entamoeba histolytica</u> , tissue culture, propagation, antibiotic, <u>Proteus</u> spp., axenic.			
10. SUPPORTING PROJECTS <div style="text-align: center;">Not Applicable</div>			
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <div style="display: flex; justify-content: space-between;"> <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet </div>		12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES <div style="display: flex; justify-content: space-between;"> <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet </div>	
DA FORM 1309R <div style="text-align: center; font-size: small;">1 Jun 63</div>		PREVIOUS EDITIONS ARE OBSOLETE	
		PAGE 1 of ____	

ACCESSION NO.

36204

ARMY RESEARCH TASK REPORT

13. PROJECT, TASK OR
SUBTASK NUMBER

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
3	A	0	1	3	0	0	1	A	8	1	4	0	1	0	9			

14. DATE OF REPORT (30-33)

30	31	32	33	34
0	6	6	4	4

15. SECURITY OF WORK (34)

16. TYPE OF REPORT

35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53		
2																	0	6	6	4

17. SCIENTIFIC FIELD

 a. Topical Classific. (56-61)
 b. Functional Class (62-64)

56	57	58	59	60	61	62	63	64
0	1	0	2	0	6			

18. OSD CLASSIFICATION

(65-66)

19. R&D CATEGORY (67)

65	66	67
B	R	1

20. CONTRACT NUMBER

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
D	A															

21. GRANT NUMBER

28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46
D	A												G					

22. ESTIMATED COMPLET.
DATES

47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70
1	0	6	6	4	2					3					4					5			

23. PRIORITY (11-14)

24. PROGRAM ELEMENT
(15-26)

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
			1	6	1	1	3	0	0	1	1				

25. CMR&D CODES

27	28	29	30	31	32	33	34	35
N	/	A						

26. CDOG REFERENCE

a. Paragraph No. (36-44)

b. Functional Group (45)

36	37	38	39	40	41	42	43	44	45
									6

27. FUNDING

a. Est. Total Cost (11-15)

b. % Spent Intern. (16-18)

" " Extern. (19-21)

c. Total Obligation (22-26)

d. Progrmd. Cur. FY (27-33)

e. " " " +1 (34-40)

f. " " " +2 (41-47)

g. " " " +3 (48-54)

h. " " " +4 (55-61)

i. " " " +5 (62-68)

j. " " " +6 (69-75)

k. Total Man Years of

Effort

(76-78)

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
					1			2							
27	28	29	30	31	32	33	34	35	36	37	38	39	40		
41	42	43	44	45	46	47	48	49	50	51	52	53	54		
55	56	57	58	59	60	61	62	63	64	65	66	67	68		
69	70	71	72	73	74	75	76	77	78						

DA FORM 1309R
1 June 63

Previous Editions are Obsolete

Page 2 of _____

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

(A) Schneider, H. S., Ph.D., Dept of Applied Immunology, Div of Communicable Diseases & Immunology, WRAIR, WRAMC, Washington, D. C., 20012

576-3010 or Interdepartmental Code 198, Ext. 3010

49

(A) Houston, F. M., B. S., Veterans Administration Central Laboratory for Clinical Pathology and Research, Dept of Bacteriology, Div of Communicable Diseases & Immunology, WRAIR, WRAMC, Washington, D. C., 20012

576-3758 or Interdepartmental Code 198, Ext 3758

49

REPORTS: Annual Progress Report, Walter Reed Army Institute of Research, 1 July 1963 - 30 June 1964.

For previous years refer to Incl 10, Ltr, MEDEC-Z, HQ, WRAIR, 1 July 1963, subject, In-House Laboratory Director's Program, Reports Control Symbol CSCRD-03(OT)-72 to Asst Secty of Army (R&D).

ANNUAL PROGRESS REPORT (FINAL)

Project No. 3A013001A814

Title: In-House Laboratory Initiated R&D

Task No. 01

Title: In-House Lab Initiated R&D

Subtask No. 09

Title: Growth of Entamoeba histolytica
in tissue cultures

Description: The primary purpose of this investigation has been an attempt to develop a tissue culture technic for axenic cultivation of Entamoeba histolytica.

Progress: Studies on Entamoeba histolytica were initiated to determine whether a monolayer tissue culture system would support the growth of this amoeba. Such a system could be used to (1) attempt to produce axenic cultures of this amoeba; (2) study the interaction of amoebae and tissue in the presence or absence of various bacteria; (3) perform immunologic studies with E. histolytica; and (4) test therapeutic agents in this system. Data compiled to date show that of three lines used (Hela, Chang human liver, and rhesus monkey kidney), rhesus monkey kidney monolayer cell cultures yield the best growth of this amoeba, perhaps due to its inherent resistance to bacterial destruction. All strains tested to date (11) can be grown on this tissue with an overlay of 99 per cent bovine amniotic fluid, one per cent male mouse liver, and 100-250 mcg/ml neomycin. Amoebae have been continuously cultured in this medium with a resistant Proteus spp. for 40 days when serially transferred every other day.

Although phosphotungstic acid hematoxylin staining has revealed normal amoebae, there is a gradual cessation of growth of the amoebae in tissue culture. Nutritional supplements such as hydrolyzed nucleic acids, nucleotides, nucleosides, and cholesterol are being used to determine their effect on growth and longevity. Numerous attempts to eliminate all bacteria without harm to viability of the amoebae have been unsuccessful. All attempts to date have also failed to reveal an additional strain of E. histolytica with antibiotic sensitive concomitant bacterial flora so that axenic or monocontaminated cultures could be obtained. Moreover, the unsuitability of many primary rhesus monkey kidney cultures due to destructive simian viral agents have compounded the problem. Significant progress in these studies would appear to be directly dependent on the acquisition of a culture of E. histolytica with an antibiotic susceptible concomitant bacterial flora and a reliable cell culture line.

Summary and Conclusions: Entamoeba histolytica, monocontaminated with an antibiotic resistant Proteus spp. has been continuously cultured on rhesus monkey kidney tissue culture for periods up to 40 days. All attempts to date to eliminate the proteus contaminant have been unsuccessful as have attempts to find a strain of amoeba free of resistant bacterial flora.

ARMY RESEARCH TASK REPORT				REPORTS CONTROL SYMBOL CSCRD-4(R2)	
ACCESSION NUMBER 36205			PROJECT, TASK, OR SUBTASK NO. 3A013001A8140110		
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D. C., 20315			2. FUNDING AGENCY Army Medical R&D Command Office of The Surgeon General Washington, D. C., 20315		
3. CONTRACTING AGENCY NA			4. CONTRACTOR AND/OR GOV'T LABORATORY A Walter Reed Army Inst of Rsch Walter Reed Army Medical Center Washington, D. C., 20012 723-1000, Ext 3552		
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Taylor, R. L., Major, MSC, Dept of Bacteriology, Div of Communicable Diseases & Immunology, WRAIR, WRAMC, Washington, D. C., 20012 576-3758 or Interdepartmental Code 198, Ext 3758 See Continuation Sheet 49					
6. TITLE OF: PROJECT <input type="checkbox"/> TASK <input type="checkbox"/> SUBTASK <input checked="" type="checkbox"/> Metabolic activities of the pathogenic fungi (U)					
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964					
8. RESUME (U) Preliminary experimental evidence indicates that the yeast phase and mycelial phase of <u>Sporotrichum schenckii</u> exhibit little difference in their ability to utilize carbohydrates. The yeast phase of <u>Histoplasma capsulatum</u> utilizes glucose and galactose without adaptation whereas the mycelial phase must induce enzymes for galactose utilization. Present Status: Completed.					
9. KEY WORDS <u>Sporotrichum schenckii</u> , <u>Histoplasma capsulatum</u> , carbohydrate utilization, yeast phase, mycelial phase, induction.					
10. SUPPORTING PROJECTS Not Applicable					
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet			12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet		
DA FORM 1309R 1 Jun 63 PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of 1					

ACCESSION NO.

36205

ARMY RESEARCH TASK REPORT

13. PROJECT, TASK OR
SUBTASK NUMBER

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
3	A	0	1	3	0	0	1	A	8	1	4	0	1	1	0			

14. DATE OF REPORT (30-33)

30	31	32	33	34
0	6	6	4	4

15. SECURITY OF WORK (34)

16. TYPE OF REPORT

35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53		
2																	0	6	6	4

17. SCIENTIFIC FIELD

 a. Topical Classific. (54-61)
 b. Functional Class (62-64)

56	57	58	59	60	61	62	63	64
0	1	0	2	0	6			

18. OSD CLASSIFICATION

(65-66)

19. R&D CATEGORY (67)

65	66	67
B	R	1

20. CONTRACT NUMBER

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
D	A															

21. GRANT NUMBER

28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46
D	A												G					

22. ESTIMATED COMPLET.
DATES

47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70
1	0	6	6	4	2					3					4					5			

23. PRIORITY (11-14)

24. PROGRAM ELEMENT
(15-26)

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
			1	6	•	1	1	•	3	0	•	0	1	•	1

25. CMR&D CODES

27	28	29	30	31	32	33	34	35
N	/	A						

26. CDOG REFERENCE

a. Paragraph No. (36-44)

b. Functional Group (45)

36	37	38	39	40	41	42	43	44	45
									6

27. FUNDING

a. Est. Total Cost (11-15)

b. % Spent Intern. (16-18)

c. Total Obligation (22-26)

d. Program. Cur. FY (27-33)

e. " " " +1 (34-40)

f. " " " +2 (41-47)

g. " " " +3 (48-54)

h. " " " +4 (55-61)

i. " " " +5 (62-68)

j. " " " +6 (69-75)

k. Total Man Years of

Effort

(76-78)

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
					1			2						
27	28	29	30	31	32	33	34	35	36	37	38	39	40	
41	42	43	44	45	46	47	48	49	50	51	52	53	54	
55	56	57	58	59	60	61	62	63	64	65	66	67	68	
69	70	71	72	73	74	75	76	77	78					

DA FORM 1309R
June 63

Previous Editions are Obsolete

Page 2 of _____

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

(A) Rust, J. H., Jr., Ph.D., Dept of Bacteriology, Div of
Communicable Disease & Immunology, WRAIR, WRAMC, Washington,
D. C., 20012
576-3758 or Interdepartmental Code 198, Ext 3758

49

REPORTS. Annual Progress Report, Walter Reed Army Institute of
Research, 1 July 1963 - 30 June 1964.

For previous year see Incl 9, Ltr, MEDEC-Z, HQ, WRAIR,
1 July 1963, subject: In-House Laboratory Director's
Program, Reports Control Symbol CSCRD-03(OT)-72, to Asst
Secty of Army (R&D).

ANNUAL PROGRESS REPORT (FINAL)

Project No. 3A013001A814

Title: In-House Laboratory Initiated R&D

Task No. 01

Title: In-House Lab Initiated R&D

Subtask No. 10

Title: Metabolic activities of
pathogenic fungi

Description: Metabolic studies of both the yeast phase and mycelial phase of Histoplasma capsulatum and Sporotrichum schenckii were initiated in an effort to obtain definitive information on the metabolic capabilities of these pathogenic fungi.

Progress: Further work in this area has been temporarily suspended since Major Taylor has been attending the Medical Field Service School for the past four months and Dr. Rust has currently been devoting full time activity to the meningococcal problem.

Experiments on the yeast and mycelial phase of Sporotrichum schenckii have been in progress with respect to carbohydrate utilization and inductive enzyme formation. It has been demonstrated that there is little or no difference between the yeast and mycelial phase organisms in their ability to utilize carbohydrates. The rate of oxidative dissimilation of xylose, mannose, rhamnose, dextrose, maltose and cellobiose by resting yeast phase cells is proportional to the rate of growth of these cells when these carbohydrates are utilized as the carbon source in growth media. Starved cells in the mycelial phase, in contrast to starved cells in the yeast phase, oxidatively metabolized most of the carbohydrates tested at a rate considerably greater than the rate observed in non-starved cells. With the methods devised for growing and manipulating both yeast and mycelial phase S. schenckii, we have now begun to examine the highly pathogenic mycotic agents. Preliminary experiments on Histoplasma capsulatum have indicated that the yeast phase utilizes glucose and galactose with no adaptation necessary. However, mycelial phase cultures grown on glucose may have to produce enzymes necessary for the utilization of galactose since an adaptation period appears to be necessary.
The influence of the induction of enzymes on the growth of the yeast phase of S. schenckii has been studied.

Summary and Conclusions: The yeast and mycelial phase of S. schenckii utilize carbohydrates equally well. Enzyme induction is necessary for the mycelial phase of H. capsulatum to utilize galactose, but not the other carbohydrates studied.

ARMY RESEARCH TASK REPORT				REPORTS CONTROL BY CICRD-4/R2
ACCESSION NUMBER 36206		PROJECT, TASK, OR SUBTASK NO. 3A013001A81140111		
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D. C., 20315		2. FUNDING AGENCY Army Medical R&D Command Office of The Surgeon General Washington, D. C., 20315		
3. CONTRACTING AGENCY NA		4. CONTRACTOR AND/OR GOV'T LABORATORY A Walter Reed Army Inst of Resch Walter Reed Army Medical Center Washington, D. C., 20012 723-1000, Ext 3552		
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Lowenthal, Joseph P., ScD., Department of Biologics Research Div of Comm Dis & Imm, WRAIR, WRAMC, Washington, D. C., 20012 576-5208 or Interdepartmental Code 198, Ext 5208 See Conting. Sheet				
6. TITLE OF: PROJECT <input type="checkbox"/> TASK <input type="checkbox"/> SUBTASK <input checked="" type="checkbox"/> Kinetics of freeze-drying of biological mixtures.				
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964				
8. RESUME (U) Installation of a new chamber-type freeze-dryer, which will permit the freeze-drying of experimental pilot scale production lots of biologicals under strictly controlled conditions, has been completed. Studies were initiated on the programming of the freeze-drying cycles of several biological products of current interest. However, this work was temporarily interrupted because of other commitments.				
9. KEY WORDS Freeze-drying, biological products, maintenance of biological activity, residual moisture, biological mixtures, inert carriers.				
10. SUPPORTING PROJECTS Not applicable.				
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet		12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet		
DA FORM 1309R 1 June 63		PREVIOUS EDITIONS ARE OBSOLETE		PAGE 1 of ____

ACCESSION NO.		ARMY RESEARCH TASK REPORT	
36206			
13. PROJECT, TASK OR SUBTASK NUMBER	11 22 23 24 25 26 27 28 29 3 A 0 1 3 0 0 1 A 8 1 4 0 1 1 1 1		
14. DATE OF REPORT (30-33)	30 33 34 0 6 6 4 4		
15. SECURITY OF WORK (34)			
16. TYPE OF REPORT	35 36 47 48 49 50 51 52 53 1 1 1 1 1 1 1 1 1 1 1 1		
17. SCIENTIFIC FIELD a. Topical Classific. (56-61) b. Functional Class (62-64)	56 61 62 64 0 1 0 6 0 8 1 1 1		
18. OSD CLASSIFICATION (65-66)	65 66 67 A R 1		
19. R&D CATEGORY (67)			
20. CONTRACT NUMBER	11 12 13 14 15 17 18 21 22 26 27 D A 1 1 1 1 1 1 1 1 1 1		
21. GRANT NUMBER	28 29 30 33 34 35 36 38 39 40 41 45 46 D A 1 1 1 1 1 1 1 1 1 1 1 1		
22. ESTIMATED COMPLET. DATES	47 51 52 56 57 61 62 66 67 7 1 C O N T 2 3 4 5 6 7 8 9		
23. PRIORITY (11-14)	11 14 15 26 1 1 1 1 1 1 1 1		
24. PROGRAM ELEMENT (15-26)			
25. CMR&D CODES	27 29 30 32 33 35 N / A 1 1 1 1 1		
26. CDOG REFERENCE a. Paragraph No. (36-44) b. Functional Group (45)	36 39 40 41 42 43 44 45 1 1 1 1 1 1 1 6		
27. FUNDING a. Est. Total Cost (11-15) b. % Spent Intern. (16-18) " " Extern. (19-21) c. Total Obligation (22-26) d. Program. Cur. FY (27-33) e. " " " +1 (34-40) f. " " " +2 (41-47) g. " " " +3 (48-54) h. " " " +4 (55-61) i. " " " +5 (62-68) j. " " " +6 (69-75) k. Total Man Years of Effort (76-78)	11 15 16 18 19 21 22 26 1 1 1 1 1 1 1 1 27 28 29 33 34 35 36 40 1 1 1 1 1 1 1 1 41 42 43 47 48 49 50 54 1 1 1 1 1 1 1 1 55 56 57 61 62 63 64 68 1 1 1 1 1 1 1 1 69 70 71 75 76 78 1 1 1 1 1 1		

DA FORM 1309R
1 June 63

Previous Editions are Obsolete

Page 2 of _____

ACCESSION NUMBER

36206

ARMY RESEARCH TASK REPORT**Continuation Sheet****PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:**

(A) Altieri, P. L., B. S., Dept of Biologics Research
Div of Comm Dis & Imm, WRAIR, WRAMC, Washington, D. C., 20012
576-5232 or Interdepartmental Code 198, Ext 5232 49

(A) Berman, S. L., PhD., Dept of Biologics Research
Div of Comm Dis & Imm, WRAIR, WRAMC, Washington, D. C., 20012
576-5232 or Interdepartmental Code 198, Ext 5232 49

REPORTS. Annual Progress Report, Walter Reed Army Institute
of Research, 1 July 1963 - 30 June 1964.

ANNUAL PROGRESS REPORT

Project No. 3A013001A814	Title: In-House Laboratory Initiated R&D
Task No. 01	Title: In-House Laboratory Initiated R&D
Subtask No. 11	Title: Kinetics of freeze-drying of biological mixtures.

Description: The purpose of this program is to examine the interaction of components of biological mixtures during differing cycles of freeze-drying. Of particular interest is the apparent protective effect of inert materials on the maintenance of the biological activity of certain viruses and bacteria.

Progress:

Installation of a new chamber-type freeze-dryer with a 30-liter condensing coil capacity was completed during this period. This unit, which can be operated by manual or automatic controls, will permit the freeze-drying of biologicals under strictly controlled conditions. For each product, the cycle that produces the best results with regard to potency, residual moisture and appearance, as determined by operation of the freeze-dryer by manual controls, can be cut into a cam for automatic control. Identical freezing and drying cycles can then be employed for additional lots of the same product. The incorporation of an internal stoppering mechanism permits closure of the bottles containing the dried product under vacuum directly in the freeze-drying cabinet. This will eliminate the absorption of moisture to the dried product, a problem which was previously experienced with the old freeze-dryer when it was necessary to transfer the unsealed product bottles from the drying chamber to a separate stoppering chamber.

After installation, the operation of the freeze-dryer was thoroughly studied to determine whether the equipment would perform as specified. As a result of these studies, several alterations were made by the manufacturer to simplify the operation.

Experimental studies on the optimal freezing and heating cycles for several biological products of current interest were initiated. These studies were interrupted because of other commitments, but will be resumed when time permits.

Summary and Conclusions:

Installation of a new chamber-type freeze-dryer, which will permit the freezing and drying of biologicals under controlled conditions, was

completed during this period. The equipment provides for either manual or automatic control of the freezing and drying cycles, and contains an internal stoppering mechanism for closure of the product bottles directly in the cabinet.

Following a thorough check of the operation of the freeze-dryer, studies on the optimal freezing and drying cycles for several biological products were initiated. These studies were interrupted because of other commitments, but will be resumed when time permits.

ARMY RESEARCH TASK REPORT		REPORTS CONTROL SYMBOL CSCRD-4(R3)
ACCESSION NUMBER 36207		PROJECT, TASK, OR SUBTASK NO. 3A013001A8140112
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D. C., 20315	2. FUNDING AGENCY Army Medical R&D Command Office of The Surgeon General Washington, D. C., 20315	
3. CONTRACTING AGENCY NA	4. CONTRACTOR AND/OR GOV'T LABORATORY A Walter Reed Army Inst of Resch Walter Reed Army Medical Center Washington, D. C., 20012 723-1000, Ext 3552	
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Allison, James L., Capt, MSC, Dept of Molecular Biology Div of Comm Dis and Imm, WRAIR, WRAMC, Washington, D. C., 20012 576-3435 or Interdepartmental Code 198, Ext 3435 See Continuation Sheet		49
6. TITLE OF: PROJECT <input type="checkbox"/> TASK <input type="checkbox"/> SUBTASK <input checked="" type="checkbox"/> Isopycnic fractional recrystallization of soluble RNA (U)		
7. DATE OF REPORT DAY 30 MONTH June YEAR 1954		
8. RESUME (U) This project constitutes a new approach to the study of groups of closely related biological molecules and the report summarizes its application to a typical material, namely the soluble ribonucleic acids. Because they are chemically indistinguishable, the individual properties of approximately twenty kinds of molecules that comprise the soluble ribonucleic acids are unknown. However, by using fractional recrystallization in conjunction with density gradient centrifugation, it has been possible to prepare soluble RNA as a family of similar but distinguishable needle-like crystals. The s-RNA is precipitated in CsCl solutions using appropriate concentrations of AlCl ₃ , and a maximum yield is obtained by separating out the amorphous portion of the precipitate and subjecting it to further treatment. The most efficient method of describing the behavior of s-RNA crystals in the analytical ultracentrifuge is in terms of the refractive index. A method for determining the refractive index of the CsCl solutions has been utilized which also permits a knowledge of the variable concentrations at each point inside the centrifuge cell.		
9. KEY WORDS Biophysics, density gradient centrifugation, soluble RNA, RNA precipitation, fractional recrystallization, molecular biology, isopycnic, RNA crystallization		
10. SUPPORTING PROJECTS Not Applicable		
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet	12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet	
DA FORM 1309R 1 June 63 PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of _____		

ACCESSION NO.

36207

ARMY RESEARCH TASK REPORT

13. PROJECT, TASK OR
SUBTASK NUMBER

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
3	A	0	1	3	0	0	1	A	8	1	4	0	1	1	2			

14. DATE OF REPORT (30-33)

30	31	32	33	34
0	6	6	4	4

15. SECURITY OF WORK (34)

16. TYPE OF REPORT

35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55
1																				

17. SCIENTIFIC FIELD

 a. Topical Classific. (56-61)
 b. Functional Class (62-64)

56	57	58	59	60	61	62	63	64
0	1	0	1	0	0			

18. OSD CLASSIFICATION

(65-66)

19. R&D CATEGORY (67)

65	66	67
B	R	1

20. CONTRACT NUMBER

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
D	A															

21. GRANT NUMBER

28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46
D	A												G					

22. ESTIMATED COMPLET.
DATES

47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71
I	C	O	N	T	2					3					4					5				

23. PRIORITY (11-14)

24. PROGRAM ELEMENT (15-26)

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
			1	6	.	1	1	.	3	0	.	0	1	.	1

25. CMR&D CODES

27	28	29	30	31	32	33	34	35
N	/	A						

26. CDOG REFERENCE

a. Paragraph No. (36-44)

b. Functional Group (45)

36	37	38	39	40	41	42	43	44	45
									6

27. FUNDING

a. Est. Total Cost (11-15)

b. % Spent Intern. (16-18)

" " Extern. (19-21)

c. Total Obligation (22-26)

d. Program. Cur. FY (27-33)

e. " " " +1 (34-40)

f. " " " +2 (41-47)

g. " " " +3 (48-54)

h. " " " +4 (55-61)

i. " " " +5 (62-68)

j. " " " +6 (69-75)

k. Total Man Years of
Effort (76-78)

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
					1			2							
27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42
43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58
59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74
75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90

DA FORM
1 June 63 1309R

Previous Editions are Obsolete

Page 2 of _____

PRINCIPAL & ASSOC. INVESTIGATORS - Item F, Continued:

(A) Hartman, R. E., Ph. D., Dept of Molecular Biology
Div of Comm Dis & Imm, WRATR, WRAMC, Washington, D. C., 20012
576-3435 or Interdepartmental Code 198, Ext 3435 49

REPORTS, Annual Progress Report, Walter Reed Army Institute of
Research, 1 July 1963 - 30 June 1964.

ANNUAL PROGRESS REPORT

Project No. 3A013001A814

Title: IN-HOUSE LABORATORY
INITIATED R&D

Task No. 01

Title: In-House Lab Initiated R&D

Subtask No. 12

Title: Isopycnic fractional recrystallization of soluble RNA

Description:

The development of a new method of separating closely related members of families of biopolymers, as exemplified by studies on the separation of transfer ribonucleic acids.

Progress:

The manner in which nucleic acids control the growth process and the production of specific biopolymers is one of the continuing problems that confront current research. Chemical analysis can distinguish only between those nucleic acids that contain deoxyribose and those that contain ribose. Within the latter group, however, there are already known to exist molecules that function as ribosomal RNA, messenger RNA, or transfer RNA. Hence, the existence of whole families of these special molecules has been demonstrated, but their individual separation and specific study is prerequisite to further understanding of protein synthesis, antibiotic action and control of growth. Thus far limited success has resulted from the most advanced studies with either countercurrent distribution or sensitive chromatography columns.

This project constitutes a new and original approach to the problem: the formation of crystals of a group of molecules followed by fractionation of the individual crystalline substances. Both the crystallization and the fractionation are performed in a salt solution inside the cell of a high-speed centrifuge. The effect of the centrifugal field on the salt ions in the solution forces them toward the bottom of the centrifuge cell causing the solution to become more concentrated, and consequently more dense in that part of the cell. The gradient of salt concentration varying from one part of the centrifuge cell to another is maintained only as long as the centrifuge is revolving fast enough to move the salt ions toward the bottom of the cell. When nucleic acid is placed in such a cell it will be lighter than the heavier part of the salt solution and will float near the center of the cell.

This technique of "density gradient centrifugation" of solutions of nucleic acids has been used previously but the technique has now been modified to permit its application to solid crystals, thereby, greatly extending its potential.

The procedure chosen for producing the s-RNA in solid form was that of precipitation with AlCl_3 . When s-RNA is added to a solution containing .001 M AlCl_3 in concentrated CsCl , a visible precipitate is formed which later takes on crystalline appearance as it is automatically concentrated in a centrifugal density gradient. The s-RNA precipitates may also be formed by treatment with hydrochloric acid or magnesium chloride, but in these instances the crystallization is not as efficient. Any amorphous material derived from the precipitation is apparently heterogenous, because only the crystalline portion is amenable to further fractionation into subfamilies of individual crystals with distinct differences in density.

However, additional crystals may be obtained by breaking down the amorphous structure prior to repeating the precipitation process. The amorphous RNA is dissolved in 0.10 M solution of citrate buffer and from this mixture the nucleic acid may be collected by adding two volumes of alcohol and permitting the mixture to stand at 4° . The resulting RNA gel is converted into a concentrated solution by adding a minimum amount of physiological saline. From this preparation, further RNA crystals may be obtained by repeating the treatment with aluminum chloride. Thus, the procedure makes possible the collection of crystalline material in quantities sufficiently large to permit more extensive analysis.

The equilibrium sedimentation behavior of the RNA crystals is interpreted by precise refractive index measurements with a differential refractometer. Since the salt concentrations involved in the density gradient centrifugation are on the order of 8 M or 9 M, water with its refractive index of 1.33 is not a satisfactory standard. However, practical measurements can be made with isooctane or cyclohexane since these two references with refractive indices of 1.3899 and 1.4238 respectively, bracket the desired point and permit accurate interpolation.

The refractive index of approximately 8.5 M CsCl is a first order function of concentration and is described by the equation, $c = 474.694 t - 607.743$ where c is the concentration in percent by weight of CsCl and t is the refractive index. The magnitude of the refractive index in this instance is 1.41. Ordinarily the density of RNA is so great that it will not float in CsCl which fact is most likely a reflection of the number of cesium ions associated with the molecules of RNA. In the crystallized RNA, the possibility of association is decreased and, hence, the apparent density is less. This effect permits the previously impossible centrifugal studies on RNA.

The density difference among the RNA crystals is so great that they form a band in the centrifuge with an approximate width of 0.6 cm at a centrifuge speed of 17,250 rpm. The density gradient at this speed is nearly .02 grams/cm⁴. The same material when centrifuged to equilibrium at 42,040 rpm produces a band of nucleic acid with a width of 0.10 cm, the width of the band thus correlating experimentally with the inverse of the density gradient. At speeds below 17,250 rpm the RNA band extends from the bottom of the cell to the meniscus. The concentration of the appropriate CsCl solution for optimum resolution is 59.7%.

Summary and Conclusions:

This project constitutes a new approach to the study of groups of closely related biological molecules and the report summarizes its application to a typical material, namely the soluble ribonucleic acids. Because they are chemically indistinguishable, the individual properties of approximately twenty kinds of molecules that comprise the soluble ribonucleic acids are unknown. However, by using fractional recrystallization in conjunction with density gradient centrifugation, it has been possible to prepare soluble RNA as a family of similar but distinguishable needle-like crystals. The s-RNA is precipitated in CsCl solutions using appropriate concentrations of AlCl₃, and a maximum yield is obtained by separating out the amorphous portion of the precipitate and subjecting it to further treatment.

The most efficient method of describing the behavior of s-RNA crystals in the analytical ultracentrifuge is in terms of the refractive index. A method for determining the refractive index of the CsCl solutions has been utilized which also permits a knowledge of the variable concentrations at each point inside the centrifuge cell.

ARMY RESEARCH TASK REPORT			REPORTS CONTROL SYMBOL CSCRD-6(R2)	
ACCESSION NUMBER 36208		PROJECT, TASK, OR SUBTASK NO. 3A013001A8140113		
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D. C., 20315		2. FUNDING AGENCY Army Medical R&D Command Office of The Surgeon General Washington, D. C., 20315		
3. CONTRACTING AGENCY NA		4. CONTRACTOR AND/OR GOV'T LABORATORY A Walter Reed Army Inst of Rsch Walter Reed Army Medical Center Washington, D. C., 20012 723-1000, Ext 3552		
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Felsenfeld, O., Lt.Col., MC, Department of Experimental Pathology WRAIR, WRAMC, Washington, D. C., 20012 576-3053 or Interdepartmental Code 198, Ext 3053 See Continuation Sheet 49				
6. TITLE OF: PROJECT <input type="checkbox"/> TASK <input type="checkbox"/> SUBTASK <input checked="" type="checkbox"/> Relapsing fever (U)				
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964				
8. RESUME (U) A Probably new <u>Borrelia</u> was found in ticks received from Prof. A. Rafyi, who discovered a new subspecies of <u>Ornithodoros parkeri</u> (Cooley, 1936) in California. This <u>Borrelia</u> differed from the typical <u>Borrelia parkeri</u> (Davis, 1942) by multiplying slowly, causing very few relapses in young rats, and by its failure to invade the central nervous system. None of the 60 guinea pig brains showed morphological changes due to <u>Borrelia</u> nor could such organisms be visualized by staining methods in them. <u>B. parkeri</u> and <u>B. turicatae</u> were compared with this organism. Agar gel diffusion technic and analysis of the antigen-antibody reactions with the aid of agar gel electrophoresis did not yield completely uniform results, due to the antigenic variations of <u>Borreliae</u> during relapses. It seems, however, that there are differences at least in one minor antigen which may be typical for the new subtype of <u>Borrelia</u> . The major common antigenic factor of <u>Borreliae</u> permits to diagnose such infections by the fluorescent antibody technic, using sera from infected mammals and an animal blood smear containing <u>Borrelia</u> of related species, as done by Sadun <u>et al.</u> in trypanosomiasis. Status: completed.				
9. KEY WORDS Borrelia, relapsing fever, infection, immunology, bacteriology, serology.				
10. SUPPORTING PROJECTS Not Applicable				
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet		12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet		
DA FORM 1309R 1 Jun 63		PREVIOUS EDITIONS ARE OBSOLETE		PAGE 1 of ____

ACCESSION NO.

36208

ARMY RESEARCH TASK REPORT

13. PROJECT, TASK OR
SUBTASK NUMBER

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
3	A	0	1	3	0	0	1	4	8	1	4	0	1	1	3			

14. DATE OF REPORT (30-33)

30	31	32	33	34
0	6	6	4	4

15. SECURITY OF WORK (34)

16. TYPE OF REPORT

35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53		
2																	0	6	6	4

17. SCIENTIFIC FIELD

a. Topical Classific. (56-61)
b. Functional Class (62-64)

56	57	58	59	60	61	62	63	64
0	1	0	2	0	6			

18. OSD CLASSIFICATION

(65-66)

19. R&D CATEGORY (67)

65	66	67
R	R	1

20. CONTRACT NUMBER

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
D	A															

21. GRANT NUMBER

28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46
D	A												G					

22. ESTIMATED COMPLET.
DATES

47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71
1	0	6	6	4	2					3					4					5				

23. PRIORITY (11-14)

24. PROGRAM ELEMENT (15-26)

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
			1	6	1	1	3	0	0	1	1				

25. CMR&D CODES

27	28	29	30	31	32	33	34	35
N	/	A						

26. CDOG REFERENCE

a. Paragraph No. (36-44)

b. Functional Group (45)

36	37	38	39	40	41	42	43	44	45
									6

27. FUNDING

a. Est. Total Cost (11-15)

b. % Spent Intern. (16-18)

Extern. (19-21)

c. Total Obligation (22-26)

d. Program. Cur. FY (27-33)

e. " " " +1 (34-40)

f. " " " +2 (41-47)

g. " " " +3 (48-54)

h. " " " +4 (55-61)

i. " " " +5 (62-68)

j. " " " +6 (69-75)

k. Total Man Years of
Effort (76-78)

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
					1			2							
27	28	29	30	31	32	33	34	35	36	37	38	39	40		
41	42	43	44	45	46	47	48	49	50	51	52	53	54		
55	56	57	58	59	60	61	62	63	64	65	66	67	68		
69	70	71	72	73	74	75	76	77	78						

DA FORM 1309R
1 June 63

Previous Editions are Obsolete

Page 2 of _____

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

(A) Dupont, J. R., Capt., MC, Division of Neuropsychiatry
WRAIR, WRAMC, Washington, D. C., 20012
576-3594 or Interdepartmental Code 198, Ext 3594

49

REPORTS. See Incl 5, Ltr, MEDEC-Z, HQ, WRAIR, 1 July 1963, subject,
In-House Laboratory Director's Program, Reports Control Symbol CSCRD-
03 (OT)-72 to Asst Secty of Army (R&D), for previous year.

Annual Progress Report, Walter Reed Army Institute of
Research, 1 July 1963 - 30 June 1964.

ANNUAL PROGRESS REPORT (FINAL)

Project No. 3A013001A814

Title: In-House Laboratory Initiated
R&D

Task No. 01

Title: In-House Lab Initiated R&D

Subtask No. 13

Title: Relapsing fever

Description:

Study of the antigenic properties of Borreliae with special regard to improvement of diagnostic procedures and possible active immunization.

Progress:

A Borrelia subspecies first noted by Prof. A. Rafyi in the Californian variant of Ornithodoros parkeri was studied and was found to differ antigenically and biologically from the typical B. parkeri as well as from B. turicatae.

Guinea pigs and mice were not found feasible for experiments with these strains. Only young rats, 20 to 22 days old were susceptible to these Borreliae to certain extent but while B. turicatae caused at least two relapses in approximately 60% of them, only one attack was observed after inoculation with Prof. Rafyi's organism. The rats survived the infections. The number of B. parkeri (Subspec.) organisms was very low in the peripheral blood. Rabbits did not become ill but developed agglutinating, precipitating and lytic antibodies.

Eight rabbit and 17 rat immune sera, together with B. recurrentis, B. duttoni and B. persica antisera, the latter kindly supplied by Prof. Rafyi, were available. The gamma globulin of these sera did not vary significantly according to the Borrelia strain used for the immunization or infection. There were pertinent changes in the beta-2-globulin fraction.

Antigens prepared from B. parkeri subtype contained a protein fraction common to Borreliae. There were, however, 3 to 6 antigenic fractions discernible by agar diffusion methods in the antigens prepared from the Borreliae. There is a species-specific polysaccharide which shows quantitative variations in organisms isolated from different relapses. Alkali-resistant but heat-labile and trypsin-resistant substances, to be designated as HL, seemed to be responsible for the altered immunological activity in relapses. These HL substances are not identical with the species-specific polysaccharides. It appears that Borreliae with one HL are destroyed at the end of the attack.

Organisms with a modified HL multiply and introduce the next relapse. The HL antigen is protein-like. When Borreliae are acted upon by antiserum, complement and zymase, this antigen cannot be recovered from Borrelia extracts. Thus, it may be involved in borreliolysin activity.

The considerable divergence of the specific polysaccharides precludes the preparation of a polyvalent Borrelia vaccine. Further experiments with borreliolysin and related substances may, however, lead to the discovery of an efficient method to enhance resistance to Borrelia infections in man.

One paper, with Prof. A. Rafyi, "A New Strain of Borrelia from a Subspecies of Ornithodoros parkeri," is being prepared. Data for a second, "Antigenic Analysis of Some Borrelia Strains," are being readied.

Summary and Conclusions:

The study of a new subtype of Borrelia from Californian O. parkeri was completed. Antigenic analysis of this and related Borreliae showed a protein common to all Borreliae which can be stained also with the aid of the fluorescent method; polysaccharides which are fairly constant in relapse strains of the same species; and a heat-labile proteinic fraction which varies under the influence of bacteriolysin. The variations of the antigens preclude the preparation of a multivalent Borrelia vaccine.

ARMY RESEARCH TASK REPORT		REPORTS CONTROL SYMBOL CSCRD-4(RS)
ACCESSION NUMBER 36209	PROJECT, TASK, OR SUBTASK NO. 3A013001A8140114	
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D. C., 20315	2. FUNDING AGENCY Army Medical R&D Command Office of The Surgeon General Washington, D. C., 20315	
3. CONTRACTING AGENCY NA	4. CONTRACTOR AND/OR GOV'T LABORATORY A Walter Reed Army Inst of Rsch Walter Reed Army Medical Center Washington, D. C., 20012 723-1000, Ext 3552	
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Marvin, Sidney L., Lt Col MC, Dept of Neurophysiology, Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012 576-3728 or Interdepartmental Code 198, Ext 3728 See Continuation Sheet		
6. TITLE OF: PROJECT <input type="checkbox"/> TASK <input type="checkbox"/> Animal & human instrumentation by polygraphic recording for SUBTASK <input checked="" type="checkbox"/> study of stress, disease, & interrogation procedures (U)		
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964		
8. RESUME (U) Purpose: To study physiological responses of animals and humans instrumented for polygraphic recording in stress, disease, and interrogation procedures. Approach: Polygraph equipment has been developed for data processing of physiological variables. Parameters that can be currently processed by this system include the electrocardiogram for heart rate, ballistocardiogram, temperature, tissue optical density for pulse volume, respiration and pulse propagation time. Time of day and identification codes are added to these data. These measurements are recorded on magnetic tape which with appropriate programming permits computer analysis. These digitalized measurements can be monitored for "on line" readout with a digital printer during testing. Present Status: Completed		
9. KEY WORDS Polygraph, autonomic, data analysis, psychophysiology, interrogation, behavior, psychiatry "on line" readout, instrumented animals.		
10. SUPPORTING PROJECTS Not Applicable		
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES <small>See Continuation Sheet</small>	12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES <small>See Continuation Sheet</small>	
<div style="display: flex; justify-content: space-between;"> DA FORM 1309R 1 Jun 63 PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of 1 </div>		

ACCESSION NO.

36209

ARMY RESEARCH TASK REPORT

13. PROJECT, TASK OR
SUBTASK NUMBER

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
3	A	0	1	3	0	0	1	A	8	1	4	0	1	1	4			

14. DATE OF REPORT (30-33)

30	31	32	33	34
0	6	6	4	4

15. SECURITY OF WORK (34)

16. TYPE OF REPORT

35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	
2																	0	6	6	4

17. SCIENTIFIC FIELD

 a. Topical Classific. (55-61)
 b. Functional Class (62-64)

55	56	57	58	59	60	61	62	63	64
0	1	0	3	0	0				

18. OSD CLASSIFICATION

(65-66)

65	66	67
A	R	1

19. R&D CATEGORY (67)

20. CONTRACT NUMBER

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
D	A															

21. GRANT NUMBER

28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46
D	A												G					

22. ESTIMATED COMPLET.
DATES

47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70
1	0	9	6	3	2					3					4					5			

23. PRIORITY (11-14)

24. PROGRAM ELEMENT
(15-26)

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
			1	6	•	1	1	•	3	0	•	0	1	•	1

25. CMR&D CODES

27	28	29	30	31	32	33	34	35
N	/	A						

26. CDOG REFERENCE

 a. Paragraph No. (36-44)
 b. Functional Group (45)

36	37	38	39	40	41	42	43	44	45
									6

27. FUNDING

a. Est. Total Cost (11-15)

b. % Spent Intern. (16-18)

" " Extern. (19-21)

c. Total Obligation (22-26)

d. Program. Cur. FY (27-33)

e. " " " +1 (34-40)

f. " " " +2 (41-47)

g. " " " +3 (48-54)

h. " " " +4 (55-61)

i. " " " +5 (62-68)

j. " " " +6 (69-75)

k. Total Man Years of
Effort (76-78)

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
					1			2							
27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42
41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56
55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70
69	70	71	72	73	74	75	76	77	78						

DA FORM 1309R
1 June 63

Previous Editions are Obsolete

Page 2 of _____

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

(A) Shockley, R. O., E. E., Dept of Neurophysiology, Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012 576-3728 or Interdepartmental Code 198, Ext 3728; Washington School of Psychiatry, 1610 New Hampshire Ave., N. W., Washington, D. C. 49

(A) Robinson, R. E., E. E., Dept of Neurophysiology, Div of Neuropsychiatry, WRAIR, WRAMC, Washington, D. C., 20012 576-3728 or Interdepartmental Code 198, Ext 3728; George Washington University, 2029 G Street, N. W., Washington, D. C. 49

REPORTS. Annual Progress Report, Walter Reed Army Institute of Research, 1 July 1963 - 30 June 1964.

For previous years refer to Incl 2, Ltr, MEDEC-Z, HQ, WRAIR, 1 July 1963, subject, In-House Laboratory Director's Program, Reports Control Symbol CSCRD-03(OT)-72 to Asst Secty of Army (R&D).

ANNUAL PROGRESS REPORT (FINAL)

Project No. 3A013001A814	Title: IN-HOUSE LABORATORY INITIATED R&D
Task No. 01	Title: In-House Lab Initiated R&D
Subtask No. 14	Title: Animal and human instrumentation by polygraphic recording for study of stress, disease, and interrogation procedures

Description:

A polygraphic recorder for recording several physiological variables on magnetic digital tape in Binary Coded Decimal (BCD) and printing out "on line" has been developed and is in operation. Two (2) time intervals and four (4) amplitudes are recorded each cardiac cycle. These at present consist of heart rate period, pulse propagation time (time lapse from R wave of electrocardiogram to peak amplitude of peripheral pulse wave), amplitude of finger pulse wave, skin temperature and respiration; also included are time of day in hours, minutes and seconds and an identification code from 00 to 99.

The system is easily adapted for recording other variables such as systolic and diastolic blood pressure from indwelling arterial transducers, venous pressure readings, galvanic skin response and other variables reflecting autonomic changes. In addition, by using a switching circuit and sampling less frequently than every cardiac cycle a much larger number of variables can be sampled and recorded, for example, every other heart beat, every third heart beat, etc.

Besides having the "on line" printed data available for monitoring during the experiment, the data which is simultaneously recorded on the magnetic tape recorder is being processed on the IBM 1410 digital computer with a program written in Fortran II which computes the mean of each variable every ten seconds, its standard deviation and the number of data on which the computation was based. Limits have been set in the program to eliminate erroneous readings as a result of artifact. Other statistical treatment of the data is contemplated in the near future.

Progress:

The physiological variables that are currently being processed with this system include signals from electrocardiograph (EKG) electrodes for heart rate, from a photoelectric cell transducer for finger pulse volume (FPV), pulse propagation time (PPT) derived from the EKG and FPV (to be described later), skin temperature from a Yellow Springs Thermistor control probe and respiration rate from a strain guage respirometer.

Signals measured directly from these transducers have amplitudes in the range of 1 microvolt to 100 microvolts and transducer outputs are usually 500 microvolts or more. Frequency components range from less than a cycle per minute to 10,000 cycles per second most of them being between 0.2 and 500 cycles per second. Wave shapes are sinusoidal or highly complex and many signals have repetitive components.

Amplification of signals is with a band-pass to DC and most signals are amplified to approximately 1 volt with a maximum voltage gain of 1000X. The amplifiers are operated wideband. When it is necessary to filter out specific frequency components it is achieved either at the input or after initial amplification. EKG signals are amplified by a Sanborn Model 350-1000 carrier preamplifier. The finger pulse volume signal is amplified by an Electro Instruments DC amplifier. Respiration and temperature signals are amplified by Dymec 2461A DC amplifiers.

Analog to digital converters are employed to convert the continuously varying signals into discretely varying quantities. These are recorded by digital logic circuitry for storage on magnetic tape and for display with a digital printer and nixie tubes. The information contained within an analog signal is a function of its amplitude and frequency. The techniques by which the analog to digital converters determine time intervals and amplitudes is described below.

Time interval measurements are made in the peak identifier between two (2) points of zero slope on the same signal for EKG detection and between points of zero slope on two (2) different signals (EKG and FPV) for pulse propagation time determination. The first step in this process is to pass the amplified EKG and FPV signals through separate band-pass amplifiers which emphasize the signal peak and reject the noise. Noise (120 cps) originating from the ambient artificial light is reduced by a notch filter. To minimize the effect of large amplitude fluctuations encountered in some signals a logarithmic amplifier subsequent to the filter stages is employed.

After the filtering and amplification, the signals are differentiated and sliced to remove all components below a critical level. The remaining wave form is converted to a rectangular pulse by a Schmitt trigger the leading edge of which corresponds to the peak of the original waveform.

Because the signal baseline is not constant and more than one peak or high amplitude interference signal may be present the output of the Schmitt trigger drives a one shot multivibrator which is set for a time interval that is sufficiently short so as not to overlap successive signals. This is of such duration that it does overlap most of the "noise." The final signal output is thus a rectangular wave of fixed duration with a leading edge corresponding in time to the incidence of the maximum peak of the input waveform. A rectangular waveform is generated which has a duration corresponding to the time between the peak

of the R wave of the EKG and the maximum peak of the finger pulse volume waveform. Another rectangular waveform is generated for the time interval between two successive R waves.

Once the appropriate rectangular waveforms have been generated the interval is readily measured by a conventional time interval meter. The instrument utilized in this system is the Hewlett-Packard 5512 Electronic Counter. This is a solid state counter with a maximum counting rate of 300 KC and a five (5) digit inline (nixie) display. It includes a crystal frequency standard and an internal storage unit which permits the display of the previous count while the next is accumulating.

Modification of this instrument for time interval measurements involves driving the "gate flip-flop" with a rectangular waveform the duration of which is equal to the interval to be measured. The "gate flip-flop" then controls the entry of pulses from the crystal frequency standard into the counters. The number of pulses is proportional to the time interval. It is also necessary to modify the reset circuitry so that the decade counters are reset prior to each count.

A special technique is used to measure the time interval between successive R waves. A single pulse must supply both start and stop information. In order to use a single time interval meter a short, constant duration pulse is generated which stops the count, transfers the data into storage, zeros the counters, and then starts the count again. The actual measurement is equal to the true time interval less the duration of this pulse. The error interval in this equipment is 3.0 milliseconds which was determined by the transmission and settling times between the counter and the digital printer, and the cycle time of the format scanner. The counter's storage unit provides a visual display of the time interval for the previous cycle.

In making amplitude measurements to determine the maximum and minimum values of a waveform a Kintel 864A Tracking Digital Voltmeter (DVM) is used. This instrument has a sample rate of 10,000 cycles per second, automatic range and polarity, and a four (4) digit inline readout. It includes logic which permits it to track a signal to locate either a maximum or a minimum peak. The 864A has been modified to permit remote operation of the maximum-minimum control circuits and the store and print commands. The unit which controls the 864A is the DVM Control Unit.

The R wave of the EKG signal is used as a "sync" signal for the operation of the voltmeter. The "sync" signal is used to set the digital voltmeter to track for a maximum (or minimum) after a fixed time delay. This is of value in initiating tracking at a time at which the peak is most likely to be present. The output of the delay one shot drives a pulse generator which in turn forces a relay flip-flop to either the maximum or minimum position. The digital voltmeter locates the desired

peak and, after the delay period determined by a ramp generator, a pulse is generated by the store command one shot. This pulse forces the relay flip-flop to change state causing the voltmeter to track to the opposite peak. The mode is now held until the incidence of the next "sync" command so that the digital voltmeter is automatically limited to one maximum and one minimum measurement for each data cycle. This feature and the delay in the first determination reduces erroneous measurements.

A Hewlett Packard 405AR DVM with three (3) digit inline nixie readout is used to record the temperature and respiration transducer outputs alternately. The maximum sampling rate of the DVM is five (5) samples per second. After amplification of the transducer signals, the signals are fed into the inputs of a relay. The R wave of the EKG signal is used as a "sync" signal which drives a flip-flop relay. The arrival of a "sync" signal causes the relay to switch from one input to the other input. In this manner each variable is measured once per two (2) data cycles. The relay can be held in one (1) position for the continuous monitoring of either one (1) of the variables. The respiration signal is sampled at a maximum rate of once per data cycle and the computer program has been written to determine change in level of the signal as a measure of respiration rate and depth.

The Printer Scanner system includes two (2) time interval meters, two (2) high speed tracking digital voltmeters, one (1) low speed digital voltmeter, a digital clock, and a manually operated data input. The scanner couples one or all of these devices to the printer. Two (2) rows (A and B) of twelve (12) columns each are required in order to print all the physiological variables obtained in each data cycle and a third row (C) is added periodically to print the time of day. The two (2) tracking voltmeters are connected permanently to columns 1-3 and 4-6 and the remainder of the digitizing devices are displayed on columns 7-12. Inputs to these latter columns are controlled by high speed mercury wetted relays. Polarity, decimal point, and certain of the decades are omitted so that the maximum amount of significant data are displayed.

Each digitizing device has an output pulse which is coincident with the completion of the various measurements. These outputs are connected to a series of print command selector switches arranged in two (2) parallel decks. The first controls the print command for the row A data and the second controls row B data. This arrangement makes it possible for any data source to initiate the print command for either row. In practice the experimental data are arranged so that the print command is issued each time by the last variable included in a particular row. After passing through the selector switches the command pulses are standardized. They may then either be divided 2:1 or used directly to drive a monostable binary unit. The division makes it possible to print every other set of data and is of value in recording variables having a repetition rate in excess of five (5) cycles per second. When two (2) rows of data are being printed alternately,

the flip-flop dividers are synchronized together so that an A row of data is always followed by a B row. This condition is maintained by coupling a pulse from the A row flip-flop to the force input of the B row flip-flop.

The store commands which initiate the print cycles drives one shot multivibrators. These introduce delays of 3.5 milliseconds on row A and 3.0 milliseconds on row B. The purpose of these delays is to provide sufficient time for the last group of data to be entered in storage and for initiation of the print command cycle. Slightly offsetting the delays avoids erroneous triggering of subsequent stages.

The delayed outputs of the monostable binary units drive a bistable binary unit and it in turn operates the high speed relay gates. The gate sequence is arranged so that a command pulse from the A row transfers the gate from the A to the B position. It is now held in this position until the B data is entered into storage at which time it returns to the A position. The gate binary may also be manually controlled to permit operation in the following modes: A row only, B row only, and alternate.

A diode network connected to the output of the monostable binary units couples the leading edge of the output of either unit to the print command delay generator maintaining the driving pulses for the gate bistable binary separately. The delay generator provides the 2.0 millisecond delay required between the initiation of the last store command in a particular group of data and the initiation of the print command. Its output drives another monostable binary unit which generates a 0.5 millisecond print command pulse.

The high speed mercury relays gate the binary coded decimal pulses but as switching transients are present they cannot be used to transfer the store command pulses. Outputs from the A and B sections of the gate bistable binary unit act as one input of a pair of transistor AND gates. The other input is the store command pulse from the appropriate digitizing device. The output from either of the AND gates is coupled to a monostable binary unit which generates the store command pulses. One gate is required for each pair of pulses that must be switched.

The Format Generator consists of an Input Buffer, Auxiliary Core Electronics, Auxiliary Transport Electronics, a Parity Computing Circuit, three (3) Logic Converters, a Potter Transport, and a Rese Magnetic Core.

The Input Buffer gates the BCD outputs of all the digitizing devices described previously with store commands and data load commands. When all three (3) signals are present data is transferred into the Auxiliary Core Electronics where the signals are amplified and fed into the magnetic core for storage. The data from each digitizing device is fed into six (6) pin connectors - one for each character - which are relocatable on the Input Buffer. Therefore the Buffer can be programmed to accept the

output from any device with a BCD output. The format of the data is determined by the numerical order in which the inputs are arranged. The Input Buffer has 62 inputs. The Input Buffer also contains a diode matrix which is described below.

The Auxiliary Core Electronics provides the timing circuitry and control for the magnetic core in addition to the data amplifiers mentioned above. For each character of data written into the core, a data pulse, start pulse, write pulse and a load level are developed. Two lines per bit binary address information is provided to the core and to the diode matrix within the Input Buffer. The binary address information is decoded to provide a discreet output for each of the 62 positions. When data is sampled at the input of the Buffer by a matrix pulse, the data is written into the current address of the core which is numerically equal to the position of the BCD input on the Input Buffer.

The Auxiliary Transport Electronics contains a James Knight 96 KC crystal oscillator which provides the clock frequencies for the system. Dividing the 96 KC by flip-flop dividers produces the 24 KC scan and 1500 cps write clock rates. Store commands from each of the digitizing devices sets a flip-flop within this unit. The flip-flop output provides a store command gate signal to the Input Buffer. After the data has been read into the core the flip-flop is reset by a return signal from the Input Buffer. The return signal drives a one shot which relays the reset of the flip-flop until the datum has been stored. Several store commands are used to set a separate bank of flip-flops whose outputs are fed into an AND gate. When all the store commands have arrived, a signal is emitted which starts the Potter transport and changes the clock rate from 24 KC to 1500 cps. The signal is fed to the Auxiliary Core Electronics after some delay and data is read from the core. This data is amplified in the Auxiliary Transport Electronics from which it is written onto the magnetic tape. After 62 characters have been written a signal is sent from the Auxiliary Core Electronics to the Auxiliary Transport Electronics which stops the transport and switches the mode from dump core to load core.

The Rese Magnetic Core has 128 six (6) bit data positions and is controlled entirely by the Auxiliary Core Electronics. It has a read or write cycle time of 30 microseconds for each character. Access time is 10 microseconds.

Data read from the core is sampled by the Horizontal Parity Electronics. If the number of data bits is even, an extra bit is added in track C of the magnetic tape after amplification in the Auxiliary Transport Electronics. This produces an odd parity tape.

The Potter Transport is a seven (7) track Digital Tape Transport with start/stop times less than 3.0 ms. It operates at 7 1/2 ips normally, but can also operate at 3 3/4 ips. It has writing capabilities only and is controlled entirely by the Auxiliary Transport Electronics.

It can operate automatically or manually. The seven (7) track head is guaranteed IBM standard compatible and only IBM tapes are used with the head.

Data is written on the tape at a rate of 1500 cps while the transport operates at 7 1/2 ips which produces a packing density of 200 characters per inch; this is considered low density tape. An Interrecord Gap of 3/4 inch is placed between records of data. The record length is .32 inches. The time required to move the tape 1.07 inches is 0.143 seconds which is the total amount of time needed to complete a core unload cycle. This figure can also be used to drive the maximum operating rate of the system which is seven (7) cycles per second. A heart rate of 420 beats per minute could therefore be recorded with the system. It must be noted that at this rate all transducer outputs would have to be sampled within 2.67 milliseconds after the R wave of the EKG. This is the time required to completely sample all 62 buffer inputs in one scan at a 24,000 cps rate.

Logic converters are used to convert the normal 1224 BCD format peculiar to Hewlett Packard equipment into the standard 1248 BCD format used by all computers. Three (3) format counters are required, two (2) for the electronic counters and one (1) for the Hewlett Packard DVM.

Of the 62 characters which constitute a record, 38 of these are data. The remaining 24 are zeros which separate one datum from another. A Fortran format statement instructs the computer to ignore the zeros and assign addresses to the data. The Data Format is as follows:

Bits 1,2	zeros
Bits 3,4,5,6	pressure #1 minimum
Bits 7,8,9	zeros
Bits 10,11,12,13	pressure #1 maximum
Bits 14,15,16	zeros
Bits 17,18,19,20	pressure #2 minimum
Bits 21,22,23	zeros
Bits 24,25,26,27	pressure #2 maximum
Bits 28,29,30	zeros
Bits 31,32,33	respiration
Bits 34,35,36	zeros
Bits 37,38,39	temperature
Bits 40,41,42	zeros
Bits 43,44,45,46	pulse propagation time
Bit 47	zero
Bits 48,49,50,51,52,53	time of day
Bits 54,55	zeros
Bits 56,57,58,59	heart period
Bit 60	zero
Bits 61,62	identification code.

The computer program which has been written for the data on the magnetic tape computes the mean and standard deviation of each variable on the IBM 1410. Before this computation takes place an autocoder program must be run on the data to change parity from odd to even because the Fortran program requires tape data to be in even parity. After this has been accomplished the Fortran program is loaded into the computer and the edited tape is read as instructed by the program.

The mean and standard deviation are computed over a 10 second interval of time. The program records the first time of day available within the first data record and converts the time to seconds. Then a comparison is made between T_0 , and T_1 , T_2 , etc. until a time difference of 10 seconds is observed. The computer stops reading data records and begins the arithmetic computing. Each kind of data (e.g. heart period) has been located in a separate array within the core memory. Before the data can be used it must be "filtered" to eliminate bad readings due to artifact or noise. Each piece of datum of one kind is compared with an upper and a lower limit. If the reading falls outside these limits the datum is replaced by 0.0. A counter counts the number of "good" readings and it is the final tally of this counter which is used to determine the mean. After all the data have been "filtered" each array of a variable is summed and divided by the number of good readings producing the mean value. Heart rate (HR) is computed from the mean of the heart period : $HR = 1/\text{heart period} \times 60$.

The mean, standard deviation, and number of good readings are now printed for all variables along with time of day and heart rate. The above constitutes one cycle and therefore the cycle repeats for each 10 second period until all the data has been processed.

Summary and Conclusions:

This processing system has the following advantages. Specific amplitude and time intervals define the parameters of interest. They are made after the initial amplification of the signals. Transducer outputs are either calibrated to indicate absolute physical quantities or are amplified to produce a signal level which is an analog of the variable. Subsequent calibration steps are not necessary and distortion of the signals by instrument noise or drift is minimal.

"On line" readout is provided so that the investigator has a continuous check on both the experiment and the operation of the digitizing equipment. The experimental data is reduced to a compact form over long periods of time which gives it wide application to chronic physiological experiments involving humans and animals. With little modification of the equipment several experimenters can use the system on a time scheduling basis.

The majority of the elements in the system consist of standard industrial instruments and it may be readily expanded or adapted. The primary importance of the system however is that it provides data processing techniques to perform analysis within a very brief time after an experimental run. The measurement and analysis of data that formerly took a technician many days and therefore drastically limited the amount of data that could be collected and the number of experiments that could be run can with this system be measured and analyzed in a very short time at a reduced monetary cost and an increased accuracy.

The primary limitations of this system, as with other recording systems, are the artifact and noise introduced by currently available transducers when the experimental subject moves about. Computer programs however may be written to exclude most of these artifacts and identify the points in the experiment when that data is not available.

ARMY RESEARCH TASK REPORT				REPORTS CONTROL SYMBOL CSCRD-4(R2)	
ACCESSION NUMBER 36210			PROJECT, TASK, OR SUBTASK NO. 3A013001A8140115		
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D. C., 20315			2. FUNDING AGENCY Army Medical R&D Command Office of The Surgeon General Washington, D. C., 20315		
3. CONTRACTING AGENCY NA			4. CONTRACTOR AND/OR GOV'T LABORATORY A Walter Reed Army Inst of Rsch Walter Reed Army Medical Center Washington, D. C., 20012 723-1000, Ext 3552		
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Barry, K. G., Lt Col, MC, Dept of Metabolism, Div of Medicine, WRAIR, WRAMC, Washington, D. C., 20012 576-3529 or Interdepartmental Code 198, Ext 3529. See Continuation Sheet					
6. TITLE OF: PROJECT <input type="checkbox"/> TASK <input type="checkbox"/> SUBTASK <input checked="" type="checkbox"/> A correlative approach to acute renal injury. (U)					
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964					
8. RESUME (U) Experimental models for acute renal and hepatic injury in the rat have been standardized and include exsanguination hypotension, transient total ischemia, hypoxia, and homologous pigment nephropathy. Micropuncture studies of pressure and flow in individual nephrons during hemorrhage, with superimposed mannitol diuresis, and under conditions of elevated ureteral pressure have defined the loss of autoregulation of tubular and peritubular capillary pressure and indicate the importance of changes in tubular permeability as well as pre-glomerular vasomotion during hypotension. Histologic studies indicate a possible medullary shunting of blood during shock. Investigations include the possible importance of tubular obstruction during recovery from hemorrhagic shock and in the genesis of renal injury produced by homologous hemoglobin. Biochemical studies of hydrolytic ("lysosomal") and oxidative enzyme activity during ischemia and autolysis were undertaken to determine their importance in cellular necrosis. The kidney responds differently than the liver and no primary role can be assigned to these enzyme systems. Basic differences of substrate specificity were found between kidney and liver tissue. Morphologic studies on the effect of hemorrhage are being completed. Autoradiographic studies with C ¹⁴ inulin are being carried out to define changes in tubular integrity during shock.					
9. KEY WORDS Kidney failure, renal physiology, nephron, micropuncture, mannitol, liver, shock					
10. SUPPORTING PROJECTS Not Applicable					
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet			12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet		
DA FORM 1309R 1 June 63 PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of _____					

ACCESSION NO. 36210		ARMY RESEARCH TASK REPORT	
13. PROJECT, TASK OR SUBTASK NUMBER		11 22 23 24 25 26 27 28 29 3 A 0 1 3 0 0 1 A 8 1 4 0 1 1 5	
14. DATE OF REPORT (30-33)		30 33 34 0 6 6 4 4	
15. SECURITY OF WORK (34)			
16. TYPE OF REPORT		35 36 47 48 49 50 51 52 53 1	
17. SCIENTIFIC FIELD a. Topical Classific. (56-61) b. Functional Class (62-64)		56 61 62 64 0 1 0 6 0 3	
18. OSD CLASSIFICATION (65-66)		65 66 67 B R 1	
19. R&D CATEGORY (67)			
20. CONTRACT NUMBER		11 12 13 14 15 17 18 21 22 26 27 D A	
21. GRANT NUMBER		28 29 30 33 34 35 36 38 39 40 41 45 46 D A G	
22. ESTIMATED COMPLET. DATES		47 51 52 56 57 61 62 66 67 7 1 C O N T 2 3 4 5	
23. PRIORITY (11-14)		11 14 15 26 1 6 1 1 3 0 0 1 1	
24. PROGRAM ELEMENT (15-26)			
25. CMR&D CODES		27 29 30 32 33 35 N / A	
26. CDOG REFERENCE a. Paragraph No. (36-44) b. Functional Group (45)		36 39 40 41 42 43 44 45 6	
27. FUNDING a. Est. Total Cost (11-15) b. % Spent Intern. (16-18) " Extern. (19-21) c. Total Obligation (22-26) d. Programd. Cur. FY (27-33) e. " " " +1 (34-40) f. " " " +2 (41-47) g. " " " +3 (48-54) h. " " " +4 (55-61) i. " " " +5 (62-68) j. " " " +6 (69-75) k. Total Man Years of Effort (76-78)		11 15 16 18 19 21 22 26 1 2 27 28 29 33 34 35 36 40 41 42 43 47 48 49 50 54 55 56 57 61 62 63 64 68 69 70 71 75 76 78	

DA FORM 1309R
1 June 63

Previous Editions are Obsolete

Page 2 of 2

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

- (A) Glinsmann, W. H. Capt. MC, Dept of Metabolism,
Div of Medicine, WRAIR, WRAMC, Washington, D. C., 20012
576-3596 or Interdepartmental Code 198, Ext 3596 49
- (A) Weiss, B., Capt. MC, Dept of Metabolism,
Div of Medicine, WRAIR, WRAMC, Washington, D. C., 20012
576-3088 or Interdepartmental Code 198, Ext 3088 49
- (A) Beach, D. J., Capt. MSC, Dept of Metabolism,
Div of Medicine, WRAIR, WRAMC, Washington, D. C., 20012
576-3573 or Interdepartmental Code 198, Ext 3573 49

REPORTS. Annual Progress Report, Walter Reed Army Institute of
Research, 1 July 1963 - 30 June 1964.

Glinsmann, W. H.: Renal Micropuncture Studies During Exsanguination
Hypotension. Clin. Res., 12: 252, 1964.

Coen, G. and Weiss, B.: Ethylene Glycol Oxidation. Fed. Proc.,
23: 224, 1964.

ANNUAL PROGRESS REPORT

Project No. 3A013001A814	Title: In-House Laboratory Initiated R&D
Task No. 01	Title: In-House Lab Initiated R&D
Subtask No. 15	Title: A correlative approach to acute renal injury

Description:

This study of experimentally produced renal injuries in animals correlates physiologic, biochemical, and morphologic changes. It encompasses: (A) The development of reproducible models of renal injury. (B) Observation of physiologic alterations, predominantly through the use of micropuncture techniques to clarify pressure and flow relationships in individual nephrons. (C) Biochemical studies of enzyme activity both from the standpoint of maintenance of enzyme integrity as well as translocations in subcellular fractions. (D) Morphologic studies using light and electron microscopy.

Progress:

A. Models of renal injury in the rat that have been standardized are exsanguination hypotension, transient total ischemia, hypoxia, and homologous pigment nephropathy. Ethylene glycol intoxication has been looked at in a very small group of animals. The choice of this variety of insults was made since each on theoretical grounds is representative of types of injury seen clinically and is anticipated to have different modes of action.

B. Physiologic studies have primarily centered on the perfusion of individual nephrons during hemorrhage, osmotic diuresis, and ureteral obstruction. Evidence has been accumulated to support the following conclusions:

In the normal animal:

(1) The prime determinants of tubular perfusion are the arterial perfusion pressure at the glomerulus and the osmotic composition of the glomerular filtrates. Total interstitial pressure changes are relatively unimportant.

(2) Autoregulation (constancy) of tubular pressure and peritubular capillaries has been confirmed.

During hemorrhagic hypotension a variety of responses can be obtained:

(1) Rapid hemorrhage is often associated with intense neurogenic vasospasm during which time tubular perfusion falls to 0.

(2) Prolonged hypotension is associated with pre-dominantly (a) myogenic vasospasm probably mediated by catechol amines acting at a preglomerular level; and (b) a stage of irreversible shock against which relative hypothermia is partially protective. Presumably this is in part mediated by endotoxin.

(3) Profuse hemorrhage is associated with a uniform fall in tubular pressures and flow. Results demonstrate: (a) Loss of autoregulation correlates well with anuria in the non-diuretic animal. (b) Hypertonic mannitol infusion prevents anuria and is associated with increased tubular perfusion at all levels of blood pressure although glomerular filtration pressure is predominantly increased at hypotensive levels. (c) Tubular perfusion persists during anuria and is associated with increased permeability of tubules. C^{14} inulin studies employing autoradiographic techniques are being carried out to define changes in tubular permeability.

(4) Reinfusion studies have, to date, yielded no evidence for a tubular obstructive phase following hemorrhage.

C. Biochemical studies have, to date, centered in two major areas:

(1) Enzyme activities in the autolyzing kidney were studied after initial studies of ischemic necrosis yielded variable results. Autolysis at 38° C is a more easily controlled system from which basic information of mechanisms of cellular necrosis can be obtained. (a) Lysosomal enzymes cathepsin and acid phosphatase in the kidney are more labile than those of liver, show a maximal increase into the soluble fraction of cells at four hours, and the phosphatase(s) has different substrate specificity from that reported in liver tissue. (b) Soluble oxidative enzymes studied were hexokinase and glucose 6 phosphate, 6 phosphogluconic acid, malic, lactic, and isocitric dehydrogenases. Changes of less than 10 per cent were found during the first eight hours of autolysis, suggesting that the physiologic changes observed during renal ischemia-anoxia of shorter duration are not mediated by changes in these enzymes.

(2) Studies on ethylene glycol metabolism were initiated in the hope of providing information that could be used to select a blocking agent for its metabolism to the renal toxin, oxalic acid. Experiments with crude tissue homogenates revealed that the chief enzymes responsible for the first oxidative step were the same as those that oxidize alcohol (alcohol dehydrogenase and catalase). Ethanol, in relatively small concentrations, was found to be an effective inhibitor of ethylene glycol oxidation by both enzyme systems. These results provide a theoretical basis for the therapeutic use of ethanol in ethylene glycol intoxication.

D. Morphologic studies have proceeded along three major paths:

(1) Considerable technical advances in handling renal tissue have been developed which allow biopsied fragments fixed with osmic

acid solutions and embedded in epon and methacrylate plastics to be sectioned in their entirety and examined by phase contrast microscopy. This allows both improved visualization by light microscopy as well as scanning of large areas preliminary to electron microscopy.

(2) A pilot study on the effect of hypoxia (5% and 10% O₂) on liver and kidney showed no significant lasting physiologic impairment or light microscopic changes; however, electron microscopy demonstrated a reversible mitochondrial lesion during which mitochondria could be seen to undergo swelling and incorporation into single membrane limited bodies (lysosomal type). Future studies with this model during the recovery phase may yield valuable information about mitochondrial regeneration.

(3) Light microscopy of tissue taken during experiments on hemorrhage shock is in preparation and will be reviewed in the near future. Material reviewed to date shows a clear delineation of osmotic effect in the outer cortex and may imply a physiologic shunting of blood away from cortical nephrons during hypotension.

Summary and Conclusions:

The progress of the microcorrelative laboratory has to date provided basic information about physiologic, biochemical and morphologic changes in renal tissue subjected to injury. Extension of biochemical studies to analysis of single or groups of cells from various sections of the nephron population will proceed when additional equipment has been installed to control temperature and humidity. Morphologic correlation will be greatly facilitated when a pathologist joins the study. With time it is anticipated that closer correlation on single models of renal injury will advance in systematic fashion.

ARMY RESEARCH TASK REPORT			REPORTS CONTROL SYMBOL CSCRD-6(R2)	
ACCESSION NUMBER 36211		PROJECT, TASK, OR SUBTASK NO. 3A013001A8140116		
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D. C., 20315		2. FUNDING AGENCY Army Medical R&D Command Office of The Surgeon General Washington, D. C., 20315		
3. CONTRACTING AGENCY NA		4. CONTRACTOR AND/OR GOV'T LABORATORY A Walter Reed Army Inst of Resch Walter Reed Army Medical Center Washington, D. C., 20012 723-1000, Ext 3552		
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Conrad, M. E., Major, MC, Dept of Hematology Div of Medicine, WRAIR, WRAMC, Washington, D. C., 20012 576-3060 or Interdepartmental Code 198, Ext 3060 See Continuation Sheet				
6. TITLE OF PROJECT <input type="checkbox"/> TASK <input type="checkbox"/> Systemic pathologic manifestations of Korean infectious SUBTASK <input checked="" type="checkbox"/> hepatitis (U)				
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964				
8. RESUME (U) Two field teams have been organized and sent to Korea to obtain specimens from U. S. soldiers with infectious hepatitis. Specimens of stool, urine and sera were collected from each patient and were transported to CONUS where they are being used for virologic and serologic study in collaboration with the Armed Forces Institute of Pathology. Aliquots were supplied to requesting laboratories for attempted isolation of the causative organism of hepatitis. Success in this endeavor would be the first step in the development of a protective vaccine. In each study the diagnosis was established by clinical observation, laboratory studies and examination of liver biopsy specimens. Symptoms and physical findings were graded to permit comparison of the disorder observed in Korea with the clinical course of hepatitis elsewhere in the world. Serial specimens of liver, kidney and gut were obtained and prepared for light and electron microscopic examination. A nonspecific lesion of the kidney was observed involving the glomeruli and tubules with interstitial edema. Marked alteration in the villous structure of the small intestine was found with formation of granulomas in the submucosa. Anemia was common and was shown to be caused, in part, by a hemolytic process. The physiologic importance of these lesions is being investigated.				
9. KEY WORDS Hepatitis, Korea, viral isolation, kidney, intestine, anemia, pathology.				
10. SUPPORTING PROJECTS Not Applicable				
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input type="checkbox"/> NO <input checked="" type="checkbox"/> YES		12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES		
Resume See Continuation Sheet		See Continuation Sheet		
DA FORM 1309R 1 Jun 63		PREVIOUS EDITIONS ARE OBSOLETE		PAGE 1 of

ACCESSION NO.

36211

ARMY RESEARCH TASK REPORT

13. PROJECT, TASK OR
SUBTASK NUMBER

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
3	A	0	1	3	0	0	1	A	8	1	4	0	1	1	6			

14. DATE OF REPORT (30-33)

30	31	32	33	34
0	6	6	4	4

15. SECURITY OF WORK (34)

16. TYPE OF REPORT

35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53
1																		

17. SCIENTIFIC FIELD
a. Topical Classific. (54-61)
b. Functional Class (62-64)

54	55	56	57	58	59	60	61	62	63	64
0	1	0	6	0	3					

18. OSD CLASSIFICATION

65	66
B	R

19. R&D CATEGORY (47)

67
1

20. CONTRACT NUMBER

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
D	A															

21. GRANT NUMBER

28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46
D	A												G					

22. ESTIMATED COMPLET.
DATES

47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71
1	C	O	N	T	2					3					4					5				

23. PRIORITY (11-14)

24. PROGRAM ELEMENT
(15-26)

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
			1	6	1	1	3	0	0	1	1				

25. CMR&D CODES

27	28	29	30	31	32	33	34	35
N	/	A						

26. CDOG REFERENCE

a. Paragraph No. (34-44)
b. Functional Group (45)

36	37	38	39	40	41	42	43	44	45
									6

27. FUNDING

a. Est. Total Cost (11-15)

b. % Spent Intern. (16-18)

Extern. (19-21)

c. Total Obligation (22-26)

d. Program. Cur. FY (27-33)

e. " " " +1 (34-40)

f. " " " +2 (41-47)

g. " " " +3 (48-54)

h. " " " +4 (55-61)

i. " " " +5 (62-68)

j. " " " +6 (69-75)

k. Total Man Years of
Effort (76-78)

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
					1			2							
27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42
43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58
59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74
75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90

DA FORM
1 June 63 1309R

Previous Editions are Obsolete

Page 2 of _____

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

- (P) Berge, T. O., Colonel, MC, Dept of Geographic Pathology
Virology Branch, AFIP, WRAMC, Washington, D. C., 20012
576-2954 or Interdepartmental Code 198, Ext 2954 49
- (A) Weintraub, L. R., Capt, MC, Dept of Hematology
Div of Medicine, WRAIR, WRAMC, Washington, D. C., 20012
576-3060 or Interdepartmental Code 198, Ext 3060 49
- (A) Schwartz, F. D., Capt, MC, Dept of Metabolism
Div of Medicine, WRAIR, WRAMC, Washington, D. C., 20012
576-3573 or Interdepartmental Code 198, Ext 3573 49
- (A) Hartman, R. E., Ph. D., Dept of Molecular Biology
Div of CD&I, WRAIR, WRAMC, Washington, D. C., 20012
576-3435 or Interdepartmental Code 198, Ext 3435 49
- (A) Hartman, R. S., Ph. D., Dept of Molecular Biology
Div of CD&I, WRAIR, WRAMC, Washington, D. C., 20012
576-3435 or Interdepartmental Code 198, Ext 3435 49
- (A) Young, A. A., MSgt, Dept of Hematology
Div of Medicine, WRAIR, WRAMC, Washington, D. C., 20012
576-3040 or Interdepartmental Code 198, Ext 3040 49

REPORTS. Annual Progress Report, Walter Reed Army Institute of Research, 1 July 1963 - 30 June 1964.

Conrad, M. E., Schwartz, F. D., and Young, A. A.: Infectious Hepatitis--A generalized Disease. A Study of Renal, Gastrointestinal and Hematologic Abnormalities. Am. J. Med., in press.

Schwartz, F. D., Conrad, M. E., and Young, A. A.: Renal Biopsy in Infectious Hepatitis. Clin. Res., 11:212, 1963.

Conrad, M. E., Hartman, R. S. and Astaldi, G.: The Gastrointestinal Lesion in Viral Hepatitis. Ann. Int. Med., 60:723, 1964.

ANNUAL PROGRESS REPORT

Project No. 3A013001A814	Title: In-House Laboratory Initiated R&D
Task No. 01	Title: In-House Lab Initiated R&D
Subtask No. 16	Title: Systemic pathologic manifestations of Korean infectious hepatitis

Description:

A collaborative effort to isolate the causative agent(s) of infectious hepatitis and delineate the pathophysiology of this disease.

Progress:

During September 1962 a three-man team was sent to Korea to collect biologic specimens from patients with clinical hepatitis. Twenty-five U. S. servicemen admitted to the 121st Evacuation Hospital volunteered to participate. The diagnosis of infectious hepatitis was established by clinical observation and laboratory studies and was confirmed by examination of liver biopsy specimens from each volunteer. The patients were transported in a group by jet aircraft to WRGH for long-term followup studies. Specimens of stool, urine and feces were collected from each patient during acute illness and convalescence and were transported to the AFIP where they are being used for virologic and serologic studies. Aliquots were supplied to other laboratories for performance of similar studies. In addition to providing material for viral isolation work this study permitted examination of the effect of hepatitis upon various organs at intervals for one year following the onset of illness. Serial biopsy specimens of liver, gut and kidney were obtained. One year after onset of clinical hepatitis active liver disease was found in the liver specimens from two patients who were asymptomatic with normal laboratory tests. In renal biopsy specimens a non-inflammatory lesion was observed which involved the glomeruli and tubules and showed interstitial edema. An inflammatory lesion was observed in specimens of the gastric and small intestinal mucosa. Electron microscopic examination of the jejunal mucosa revealed virus-like particles within the cytoplasm of these cells. The shedding of these virus-containing cells into the lumen of the gut may explain the epidemiology of this disease.

During January 1964, a second team was sent to Korea to collect larger volumes of infected plasma from proven cases of infectious hepatitis. Specimens were collected from 18 patients and will be made available to 13 laboratories in a collaborative effort to isolate the causative organism of hepatitis. Convalescent plasma is being collected in an attempt to establish serologic methods of identifying this illness. During this current study the physiologic importance of the pathologic lesions is being investigated. In addition studies have been initiated to investigate the cause of the anemia found in patients with infectious hepatitis.

It is hoped that continuation of these studies will provide a better understanding of this disease and possibly supply an effective vaccine to protect individuals from infectious hepatitis. Collection of infective materials from various parts of the world is planned to provide a greater chance of obtaining the variety of viral agents or strains that may cause this illness.

Summary and Conclusions:

Reproducible clinical studies of infectious hepatitis were performed in Korea. These studies provide a source of materials from proven cases of infectious hepatitis for collaborative isolation work by a number of laboratories. The pathologic changes observed in the kidney, gut, bone marrow and liver were studied in patients with non-fatal infectious hepatitis.

ARMY RESEARCH TASK REPORT			REPORTS CONTROL SYMBOL CSCRD-6(R2)	
ACCESSION NUMBER 36212		PROJECT, TASK, OR SUBTASK NO. 3A013001A81140117		
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D. C., 20315		2. FUNDING AGENCY Army Medical R&D Command Office of The Surgeon General Washington, D. C., 20315		
3. CONTRACTING AGENCY NA		4. CONTRACTOR AND/OR GOV'T LABORATORY A Walter Reed Army Inst of Rsch Walter Reed Army Medical Center Washington, D. C., 20012 723-1000, Ext 3552		
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Crosby, W. H., Colonel, MC, Dept of Hematology Div of Medicine, WRAIR, WRAMC, Washington, D. C., 20012 576-3365 or Interdepartmental Code 198, Ext 3365 See Continuation Sheet 49				
6. TITLE OF: PROJECT <input type="checkbox"/> TASK <input type="checkbox"/> SUBTASK <input checked="" type="checkbox"/> Absorption and loss of radioisotopes by the gut (U)				
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964				
8. RESUME (U) Ingested radioactive substances are absorbed or pass out in the feces. The mechanisms regulating the quantity of trace elements accepted or rejected by the gut for absorption are poorly understood. Radioactive substances not lost in urine or expired air usually have a prolonged biologic lifespan and are excreted from the gut and epithelial surfaces of the body. Using iron ⁵⁹ as a model, the rate and manner by which this substance is absorbed by the intestinal mucosa and is excreted by the skin and gut were studied. The iron content within the intestinal epithelium and the rate of utilization of iron were found to be more important in regulating absorption than the size of the tissue stores. Studies of iron kinetics within the intestinal epithelium and demonstration of enhanced absorption of iron ferrous metals by iron deficient animals suggest that an acceptor mechanism exists within the small intestinal epithelial cells. Excretion of iron was shown to occur from the skin and gastrointestinal tract in a limited but selective manner. In humans these types of studies have been hampered by the large quantities of radioisotope required for study. Radioautography of tissue biopsied from sites of injection of iron ⁵⁹ , activation analysis of tissues after parenteral injection of stable isotopes and whole-body counting are being used to circumvent this problem.				
9. KEY WORDS Absorption, excretion, iron, intestine, skin, mechanism, radioactive.				
10. SUPPORTING PROJECTS Not Applicable				
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet		12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet		
DA FORM 1309R 1 June 63 PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of _____				

ACCESSION NO.

36212

ARMY RESEARCH TASK REPORT

13. PROJECT, TASK OR
SUBTASK NUMBER

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
3	A	1	3	0	0	1	A	8	1	4		0	1	1	7			

14. DATE OF REPORT (30-33)

30	31	32	33	34
0	6	6	4	4

15. SECURITY OF WORK (34)

16. TYPE OF REPORT

35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55
1																				

17. SCIENTIFIC FIELD

 a. Topical Classific. (56-61)
 b. Functional Class (62-64)

56	57	58	59	60	61	62	63	64
0	1	0	6	0	3			

18. OSD CLASSIFICATION

(65-66)

19. R&D CATEGORY (67)

65	66	67
B	R	1

20. CONTRACT NUMBER

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
D	A															

21. GRANT NUMBER

28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46
D	A												G					

22. ESTIMATED COMPLET.
DATES

47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71
1	C	O	N	T	2					3					4					5				

23. PRIORITY (11-14)

24. PROGRAM ELEMENT
(15-26)

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
			1	6	•	1	1	•	3	0	•	0	1	•	1

25. CMR&D CODES

27	28	29	30	31	32	33	34	35
N	/	A						

26. CDOG REFERENCE

a. Paragraph No. (36-44)

b. Functional Group (45)

36	37	38	39	40	41	42	43	44	45
									6

27. FUNDING

a. Est. Total Cost (11-15)

b. % Spent Intern. (16-18)

" " Extern. (19-21)

c. Total Obligation (22-26)

d. Progrmd. Cur. FY (27-33)

e. " " " +1 (34-40)

f. " " " +2 (41-47)

g. " " " +3 (48-54)

h. " " " +4 (55-61)

i. " " " +5 (62-68)

j. " " " +6 (69-75)

k. Total Man Years of

Effort

(76-78)

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
					1			2							
27	28	29	30	31	32	33	34	35	36	37	38	39	40		
41	42	43	44	45	46	47	48	49	50	51	52	53	54		
55	56	57	58	59	60	61	62	63	64	65	66	67	68		
69	70	71	72	73	74	75	76	77	78						

DA FORM
1 June 63 1309R

Previous Editions are Obsolete

Page 2 of _____

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

- (A) Conrad, M. E., Maj, MC, Dept of Hematology
Div of Med, WRAIR, WRAMC, Washington, D. C., 20012
576-3060 or Interdepartmental Code 198, Ext 3060 49
- (A) Pollack, S., Capt, MC, Dept of Hematology
Div of Med, WRAIR, WRAMC, Washington, D. C., 20012
576-3060 or Interdepartmental Code 198, Ext 3060 49
- (A) Weintraub, L. R., Capt, MC, Dept of Hematology
Div of Med, WRAIR, WRAMC, Washington, D. C., 20012
576-3060 or Interdepartmental Code 198, Ext 3060 49
- (A) Kaufman, R. M., Capt, MC, Dept of Hematology
Div of Med, WRAIR, WRAMC, Washington, D. C., 20012
576-3060 or Interdepartmental Code 198, Ext 3060 49

REPORTS: Annual Progress Report, Walter Reed Army Institute of Research, 1 July 1963 - 30 June 1964.

Pollack, S., Kaufman, R., Crosby, W. H. and Butkiewicz, J. E.:
Reducing Agents and Absorption of Iron. Nature 199: 384, 1963.

Pollack, S., Balcerzak, S. P. and Crosby, W. H.: Transferrin and
the Absorption of Iron. Blood 21: 33, 1963.

Hartman, R. S., Conrad, M. E., Hartman, R. E., Joy, R. J. T. and
Crosby, W. H.: Ferritin-Containing Bodies in Human Small Intes-
tinal Epithelium. Blood 22: 397, 1963.

Conrad, M. E. and Crosby, W. H.: Intestinal Mucosal Mechanisms
Controlling Iron Absorption. Blood 22: 406, 1963.

Wheby, M. S. and Crosby, W. H.: The Gastrointestinal Tract and
Iron Absorption. Blood 22: 416, 1963.

Crosby, W. H., Conrad, M. E. and Wheby, M. S.: The Rate of Iron
Accumulation in Iron Storage Disease. Blood 22: 429, 1963.

Crosby, W. H.: The Control of Iron Balance by the Intestinal
Mucosa. Blood 22: 441, 1963.

Weintraub, L. R., Conrad, M. E. and Crosby, W. H.: The Role of
Hepatic Iron Stores in the Control of Iron Absorption. J. Clin.
Invest. 43: 40, 1964.

Williams, H. L., Anderson, P. R., Conrad, M. E. and Crosby, W. H.:
A Rapid Method of Measuring the Iron Concentration in Serum or
Plasma. J. Lab. Clin. Med. 63: 703, 1964.

ACCESSION NUMBER

36212

ARMY RESEARCH TASK REPORT

Continuation Sheet

Weintraub, L. R., Conrad, M. E. and Crosby, W. H.: Regulation of Iron Absorption. Modern Medicine, p. 200, April 27, 1964.
(Abstract)

Weintraub, L. R., Conrad, M. E., Crosby, W. H.: Iron Loading Anemia: Treatment with Pyridoxine and Repeated Phlebotomies. Ann. Int. Med. 60: 728, 1963.

DA FORM 1309R
1 June 63

PREVIOUS EDITIONS ARE OBSOLETE

Page of _____

ANNUAL PROGRESS REPORT

Project No. 3A013001A814

Title: IN-HOUSE LABORATORY
INITIATED R&D

Task No. 01

Title: In-House Lab Initiated R&D

Subtask No. 17

Title: Absorption and loss of
radioisotopes by the gut

Description: Study of the mechanisms regulating absorption and excretion of radioactive substances.

Progress:

Using iron⁵⁹ as a model, the factors controlling absorption from the gut and excretion from the body were investigated. Radioautographs of the duodenum and jejunum obtained from normal iron replete animals at intervals after oral and intravenous doses of iron⁵⁹ showed concentration of the radioactive substance within the epithelial cells of the gut. The quantity of iron contained within these cells was increased in iron-loaded animals and decreased in iron deficiency. Since there is decreased absorption of iron in the former condition and increased absorption in the latter, the factor controlling absorption by the epithelium seemed to be intrinsic iron content of these mucosal cells. Likewise, since iron is not retained in the intestinal mucosal cells of iron-deficient animals but is sequestered in increased amounts in the epithelium of iron-loaded animals; the normal loss of the mucosal cells into the lumen of the gut provides a mechanism for selective though limited excretion.

A search was initiated to identify the factor controlling absorption of iron from the intestinal epithelium into the body. Anemia, the plasma iron concentration and the size of the body iron store were each excluded as the primary stimulus to absorption. Changes in the plasma iron turnover occurred simultaneously with increases in absorption of iron and were thought to act as a means of regulating the absorption of iron.

Study of the rate of transfer of iron from the gut lumen to the mucosal cell and from the epithelium into the body showed that both factors were important in regulation of iron absorption and seemed to indicate that there was an active transport system in the intestinal mucosa. Demonstration of competitive inhibition of iron absorption by other metals indicated that this transport system might serve as a common absorptive pathway for a variety of substances.

Serial measurement of total body radioactivity after a parenteral injection of radioiron showed that there was selective loss of iron from the body and suggested that this was regulated by body requirements. In animals radioactive iron lost from the body could be accounted for in accumulating fecal collections. Human studies showed that in addition to fecal loss of iron there was loss from the skin. Mechanisms of dermal loss have been studied by kinetic measurements and radioautography.

Summary and Conclusions:

Intestinal mechanisms regulating the quantity of iron retained by the body have been studied. Sequestration of both dietary and body iron in intestinal epithelial cells has been demonstrated and quantified. The iron deposited in these cells acts to limit absorption from the gut and serves as a method of ridding the body of excess iron stores. Evidence of an active transport system in intestinal epithelial cells was found by kinetic studies and demonstration of competitive inhibition by nonferrous metals. Excretion of iron in man occurs from the gut and skin and though limited is a selective process.

ARMY RESEARCH TASK REPORT		REPORTS CONTROL SYMBOL CSORD-6(R1)
ACCESSION NUMBER 36213		PROJECT, TASK, OR SUBTASK NO. 3A013001A8140118
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D. C., 20315	2. FUNDING AGENCY Army Medical R&D Command Office of The Surgeon General Washington, D. C., 20315	
3. CONTRACTING AGENCY Hq, US Army Medical R&D Command Office of The Surgeon General Washington, D. C., 20315	4. CONTRACTOR AND/OR GOVT LABORATORY 4 University of Maryland College Park, Maryland 927-3800, Ext. 535	
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Purdy, William C., PhD, Professor, Dept Chemistry University of Maryland, College Park, Maryland 927-3800, Ext. 535		18
6. TITLE OF: PROJECT <input checked="" type="checkbox"/> TASK <input type="checkbox"/> SUBTASK <input checked="" type="checkbox"/> Nature and stability of complexes between biologically- important compounds and micronutrients (U)		18
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964		
8. RESUME (U) The principal objective of the proposed research is to study the nature of complex formation of essential metallic coenzymes with varying pH and ionic concentrations. Secondly, a detailed study of the nature of the complexes between the metal and the enzyme will be pursued. The specific initial objectives will include: Study of metallic ions in various pH systems; Study of preformed metallic chelate compounds in similar systems; Study of the interactions between the metallic complexes and enzyme systems for which they are known to be essential. With regard to these objectives, the ultimate aim is to demonstrate the dependence of interactions between the various forms of the metal and enzymes in a series of solutions with varying pH. A second aim is to relate the biological activity of various metallic forms. Preliminary polarographic experiments using copper, zinc, manganese and cobalt in the presence of THAM(trihydroxyaminomethane) indicate that there are definite complexes formed between the metallic ions and THAM.		
9. KEY WORDS Chelation, metal complexes, metallic coenzymes, trace metals, pH requirement.		
10. SUPPORTING PROJECTS Not applicable		
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOVT AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet		12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOVT. AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet
DA FORM 1309R 1 Jun 63		PAGE 1 of 1

[illegible]

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

(A) Knoblock, E. C. , Lt Col, MSC, Division Biochemistry,
WRAIR, WRAMC, Washington, D. C. , 20012
576-3528 or Interdepartmental Code 198, Ext 3528. 49

(A) Christian, G. D. , PhD, Division Biochemistry,
WRAIR, WRAMC, Washington, D. C. , 20012
576-3528 or Interdepartmental Code 198, Ext 3527 49

REPORTS. Annual Progress Report, Walter Reed Army Institute
of Research, 1 July 1963 - 30 June 1964.

ANNUAL PROGRESS REPORT

Project No. 3A013001A814 Title: In-House Laboratory Initiated R&D

Task No. 01 Title: In-House Lab Initiated R&D

Subtask No. 18 Title: Nature and stability of complexes
between biologically-important
compounds & micronutrients.

Description: The principal objective of the proposed research is to study the nature of complex formation of essential metallic co-enzymes with varying pH and ionic concentrations. Secondly, a detailed study of the nature of the complexes between the metal and the enzyme will be pursued.

The specific initial objectives will include: Study of metallic ions in various pH systems; Study of preformed metallic chelate compounds in similar systems; Study of the interactions between the metallic complexes and enzyme systems for which they are known to be essential.

With regard to these objectives, the ultimate aim is to demonstrate the dependence of interactions between the various forms of the metal and enzymes in a series of solutions with varying pH. A second aim is to relate the biological activity of various metallic forms.

Among the principal techniques to be employed for demonstration of interaction between the metal ion and the biologically-important compound will be the electro-analytical method, polarography. This tool allows a demonstration of interactions within enzyme systems and allows an approximation of the stability of complexes and a determination of the nature of these complexes. The addition of a complexing agent to a solution of a metal ion causes a shift in the half-wave potential for that ion. The magnitude of the shift is a function of the stability constant of the complex. In addition, it is also possible to determine the number of molecules of complexing agent associated with one metal ion from a plot of the half-wave potential against the logarithm of the concentration of the complexing agent. Furthermore, if the complexing agent possesses an electroactive site, the point of attachment of the complexing agent to the metal ion can be determined.

It is also possible to employ potentiometric measurements to determine the nature and stability of complexes. Additionally, ultraviolet and infra-red spectroscopy, NMR measurements, and x-ray crystallography will be employed for the characterization of the complexes, where needed.

These methods of determining the nature and stability of the complexes will be correlated with biological laboratory studies of animals subjected to varying degrees of hemorrhagic trauma in which the pH of the blood has been altered.

Progress:

This program was initiated very late in the reporting year. Polarographic experiments have demonstrated that complex formation occurs between trihydroxyaminomethane (THAM) and the metallic ions of copper, zinc, manganese and cobalt. The stability of the complex formed is dependent on the metallic ion involved. A study of the dependence of the chelate compound on pH variation is currently in progress. Various conditions of pH and ionic concentration will be tested with additional trace metals in the near future.

Summary and Conclusions:

Metallic ions form complex ions with THAM. These complexes vary in stability according to the metal involved.

ARMY RESEARCH TASK REPORT			REPORTS CONTROL SYMBOL CSCRD-6(R2)	
ACCESSION NUMBER 36214		PROJECT, TASK, OR SUBTASK NO. 3A013001A8140119		
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D. C. 20315		2. FUNDING AGENCY Army Medical R&D Command Office of The Surgeon General Washington, D. C. 20315		
3. CONTRACTING AGENCY HQ, US Army Medical R&D Command Office of The Surgeon General Washington, D. C. 20315		4. CONTRACTOR AND/OR GOV'T LABORATORY 4 Dartmouth Medical School Hanover, New Hampshire Area Code 603, 643-3000		
27				
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Schroeder, Henry A., M.D., Assoc Prof of Physiology, Brattleboro Retreat Dartmouth Medical School, Brattleboro, Vermont Area Code 802, 254-2331 See Continuation Sheet				
43				
6. TITLE OF: PROJECT <input type="checkbox"/> TASK <input type="checkbox"/> SUBTASK <input checked="" type="checkbox"/> Metallic micronutrients and intermediary metabolism (U)				
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964				
8. RESUME (U) The purpose of this project is: (1) to study the biological effects of chromium as regards survival, reproduction, resistance to infection and various other stresses and its relation to other dietary components in promoting growth and longevity of mice and rats, and (2) to ascertain whether or not vanadium, nickel and germanium are essential micronutrients for small mammals and if so, the functions of each. The project will be conducted in collaboration with Dr. Walter Mertz of the Walter Reed Army Institute of Research. A relatively metal-free animal laboratory constructed to avoid environmental metallic contaminants is located on a remote hill in Vermont. Diets deficient in the element to be evaluated will be formulated and given to about 100 mice and 100 rats of both sexes for their lifetimes, controlled by a similar number of animals given low doses of the metal in drinking water. Microanalytical chemical methods now routinely available will monitor foods, output of metal and tissue concentrations at various intervals. Growth and survival at various ages will be measured. Deficient and non-deficient animals will be subjected to a number of graded stresses in order to measure resistance. Swimming time, anesthesia, reproduction, incidence of infections and tumors, gross and microscopic pathological changes, and other parameters will be evaluated. Concurrently, biochemical measurements will be made on the tissues of these animals.				
9. KEY WORDS Chromium, vanadium, nickel, germanium, rodents, metabolism.				
10. SUPPORTING PROJECTS Not Applicable				
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet		12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet		
DA FORM 1309R PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of ____				

ACCESSION NO. 36214		ARMY RESEARCH TASK REPORT	
13. PROJECT, TASK OR SUBTASK NUMBER	11	22	23 24 25 26 27 28 29
	3 A 0 1 3 0 0 1 A 8 1 4	0 1	1 9
14. DATE OF REPORT (30-33)	30	33	34
15. SECURITY OF WORK (34)	0 6 6 4		4
16. TYPE OF REPORT	35	36	47 48 49 50 51 52 53
	1		
17. SCIENTIFIC FIELD a. Topical Classific. (56-61) b. Functional Class (62-64)	56	61	62 64
	0 1 0 6 0 6		
18. OSD CLASSIFICATION (65-66)	65 66		67
19. R&D CATEGORY (67)	BR		1
20. CONTRACT NUMBER	11 12	13 14	15 17 18 21 22 26 27
	D A	4 9	1 9 3 M D 0 2 5 9 5
21. GRANT NUMBER	28 29	30	33 35 36 38 39 40 41 45 46
	D A		
22. ESTIMATED COMPLET. DATES	47	51	52 56 57 61 62 66 67 71
	1 0 6 6 7	2	3 4 5
23. PRIORITY (11-14)	11	14	15 26
24. PROGRAM ELEMENT (15-26)		1	6 • 1 1 • 3 0 • 0 1 • 1
25. CMR&D CODES	27 29	30 32	33 35
	N / A		
26. CDOG REFERENCE a. Paragraph No. (36-44) b. Functional Group (45)	36	39 40	41 42 43 44 45
			6
27. FUNDING a. Est. Total Cost (11-15) b. % Spent Intern. (16-18) " " Extern. (19-21) c. Total Obligation (22-26) d. Progmd. Cur. FY (27-33) e. " " " +1 (34-40) f. " " " +2 (41-47) g. " " " +3 (48-54) h. " " " +4 (55-61) i. " " " +5 (62-68) j. " " " +6 (69-75) k. Total Man Years of Effort (76-78)	11	15	16 18 19 21 22 26
		1 6	1 2 0 0 1 6
	27 28	29	33 34 35 36 40
	6 4		6 5 1 6
	41 42	43	47 48 49 50 54
	6 6		1 6
	55 56	57	61 62 63 64 68
	69 70	71	75 76 78

ACCESSION NUMBER

36214

ARMY RESEARCH TASK REPORT**Continuation Sheet**

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

(A) Mertz, Walter, M.D., Div of Biochemistry

WRAIR, WRAMC, Washington, D. C. 20012

576-3528 or Interdepartmental Code 198, Ext 3528

49

REPORTS. None

DA FORM 1309R
1 June 63

PREVIOUS EDITIONS ARE OBSOLETE

Page of _____

ARMY RESEARCH TASK REPORT				REPORTS CONTROL SYMBOL CSCRD-6(R3)	
ACCESSION NUMBER 36215			PROJECT, TASK, OR SUBTASK NO. 3A013001A8140120		
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D. C., 20315			2. FUNDING AGENCY Army Medical R&D Command Office of The Surgeon General Washington, D. C., 20315		
3. CONTRACTING AGENCY HQ, US Army Medical R&D Command Office of The Surgeon General Washington, D. C., 20315			4. CONTRACTOR AND/OR GOV'T LABORATORY 4 Georgetown University 1834 Connecticut Avenue Washington, D. C. 333-2000. 49		
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Bellanti, Joseph A., M.D., Asst Prof of Pediatrics and Microbiology Georgetown University School of Medicine, Washington, D. C. 333-2000, Ext 504 49 See Continuation Sheet					
6. TITLE OF: PROJECT <input type="checkbox"/> TASK <input type="checkbox"/> Physicochemical studies of the biologic significance of SUBTASK <input checked="" type="checkbox"/> antiviral antibody (U)					
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964					
8. RESUME (U) This project is aimed at obtaining a definitive study of biological significance of the various types of antiviral antibodies so as to determine whether differences in the sequential appearance of antibodies induced by viral antigens are related to the nature of the virus, and to define and characterize the relationship of local antibodies in respiratory sections which have been shown to be associated with gamma-1A globulins, to humoral antibody. This investigation represents follow-up on the results of preliminary work at WRAIR on <u>in vitro</u> marker tests for antibodies. By determining whether differences in the character of antibody following infection and immunization exist, it is possible that a method for evaluation of vaccine effectiveness without repeated field trials may be developed. <div style="text-align: right; margin-top: 20px;">P. 10, 11, 12</div>					
9. KEY WORDS Virus, antibody, globulins, infection, immunization, respiratory secretion.					
10. SUPPORTING PROJECTS Not Applicable					
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet			12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet		
DA FORM 1 June 63 1309R		PREVIOUS EDITIONS ARE OBSOLETE		PAGE 1 of _____	

ACCESSION NO.

36215

ARMY RESEARCH TASK REPORT

13. PROJECT, TASK OR
SUBTASK NUMBER

11	22	23 24	25 26	27 28	29
3 A 0 1 3 0 0 1 A 8 1 4	0 1	2 0			

14. DATE OF REPORT (30-33)

30	33	34
0 6 6 4	4	

15. SECURITY OF WORK (34)

16. TYPE OF REPORT

35	36	47 48	49 50	51	52	55
1						

17. SCIENTIFIC FIELD

 a. Topical Classific. (56-61)
 b. Functional Class (62-64)

56	61	62	64
0 1 0 6 0 2			

18. OSD CLASSIFICATION

(65-66)

19. R&D CATEGORY (67)

65 66	67
5 F	1

20. CONTRACT NUMBER

11 12	13 14	15	17	18	21	22	26	27
D A	4 0	1 0 3		M D		0 2 6 3 3		

21. GRANT NUMBER

28 29	30	33	34 35	36	38	39 40	41	45	46
D A							G		

22. ESTIMATED COMPLET.
DATES

47	51	52	56	57	61	62	66	67	71
1 0 6 6 9	2			3		4		5	

23. PRIORITY (11-14)

24. PROGRAM ELEMENT
(15-26)

11	14	15	26
		6 • 1 1 • 3 0 • 0 • 1	

25. CMR&D CODES

27	29	30	32	33	35
1	2				

26. CDOG REFERENCE

a. Paragraph No. (36-44)

b. Functional Group (45)

36	39	40	41 42	43 44	45
					6

27. FUNDING

a. Est. Total Cost (11-15)

b. % Spent Intern. (16-18)

" " Extern. (19-21)

c. Total Obligation (22-26)

d. Progrmd. Cur. FY (27-33)

e. " " " +1 (34-40)

f. " " " +2 (41-47)

g. " " " +3 (48-54)

h. " " " +4 (55-61)

i. " " " +5 (62-68)

j. " " " +6 (69-75)

k. Total Man Years of
Effort

(76-78)

11	15	16	18	19	21	22	26
	2	1		2 0 0			7 2
27 28	29	33	34 35	36	40		
6 4		2	6 5		3 4		
41 42	43	47	48 49	50	54		
6 6		3 4	6 7		3 4		
55 56	57	61	62 63	64	68		
6 8		3 5					
69 70	71	75	76	78			

ACCESSION NUMBER

36215

ARMY RESEARCH TASK REPORT**Continuation Sheet**

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

(A) Artenstein, M.S., Capt, MC, Dept of Virus Diseases,
Div of Communicable Disease & Immunology,
WRAIR, WRAMC, Washington, D. C. 20012

576-3757 or Interdepartmental Code 198, Ext 3757

49

REPORTS. None

DA FORM 1309R
1 June 63

PREVIOUS EDITIONS ARE OBSOLETE

Page of _____

ARMY RESEARCH TASK REPORT		REPORTS CONTROL SYMBOL CSCRD-6(R2)
ACCESSION NUMBER <div style="text-align: center; font-size: 1.2em;">36216</div>	PROJECT, TASK, OR SUBTASK NO. DA 0 13001A814 01 21	
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D. C., 20315	2. FUNDING AGENCY Army Medical R & D Command Office of The Surgeon General Washington, D. C., 20315	
3. CONTRACTING AGENCY <div style="text-align: center;">NA</div>	4. CONTRACTOR AND/OR GOV'T LABORATORY A Walter Reed Army Inst Rsch Walter Reed Army Medical Center Washington, D. C., 20012 723-1000, Ext 3552	
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Sadun, E. H., Sc.D., Dept of Medical Zoology, Division of Comm Dis & Immunol, WRAIR, WRAMC, Wash. D. C., 20012 576-3308 or Interdepartmental Code 198, Ext 3308 See continuation sheet		
6. TITLE OF: PROJECT <input type="checkbox"/> TASK <input type="checkbox"/> <u>Spirometra mansonoides</u> infection and glucose SUBTASK <input checked="" type="checkbox"/> <u>metabolism</u> (U)		
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964		
8. RESUME' (U) Infection in mice with the sparganum of <u>Spirometra mansonoides</u> produces abnormal increases in weight. A glucose tolerance test for mice was developed to determine the influence of this infection on the carbohydrate metabolism of the host. Experiments were conducted to examine some of the variables which may alter the glucose removal rates and to study the influence of this infection in altering the glucose removal rate in mice at regular intervals. Attempts to determine the biochemical mechanism of this phenomenon revealed that extracts of spargana produced a stimulation of glucose oxidation. Greatest activity was found in the water soluble fraction of the worms after extraction with acetone and methanol.		
9. KEY WORDS Sparganosis, spirometra, glucose tolerance, carbohydrate metabolism, obesity, cestodes.		
10. SUPPORTING PROJECTS Not applicable		
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet	12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet	
DA FORM 1309R 1 June 63 PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of _____		

ACCESSION NO.

36216

ARMY RESEARCH TASK REPORT

13. PROJECT, TASK OR
SUBTASK NUMBER

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
3	A	0	1	3	0	0	1	A	8	1	4	0	1	2	1			

14. DATE OF REPORT (30-33)

30	31	32	33	34
0	6	6	4	4

15. SECURITY OF WORK (34)

16. TYPE OF REPORT

35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55
1																				

17. SCIENTIFIC FIELD

- a. Topical Classific. (56-61)
b. Functional Class (62-64)

56	57	58	59	60	61	62	63	64
0	1	0	1	0	0			

18. OSD CLASSIFICATION

(65-66)

19. R&D CATEGORY (67)

65	66	67
B	R	1

20. CONTRACT NUMBER

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
D	A															

21. GRANT NUMBER

28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46
D	A												G					

22. ESTIMATED COMPLET.
DATES

47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71
1	C	O	N	T	2					3					4					5				

23. PRIORITY (11-14)

24. PROGRAM ELEMENT
(15-26)

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
			1	6	•	1	1	•	3	0	•	0	1	•	1

25. CMR&D CODES

27	28	29	30	31	32	33	34	35
N		A						

26. CDOG REFERENCE

- a. Paragraph No. (36-44)
b. Functional Group (45)

36	37	38	39	40	41	42	43	44	45
									6

27. FUNDING

- a. Est. Total Cost (11-15)
b. % Spent Intern. (16-18)
" " Extern. (19-21)
c. Total Obligation (22-26)
d. Programd. Cur. FY (27-33)
e. " " " +1 (34-40)
f. " " " +2 (41-47)
g. " " " +3 (48-54)
h. " " " +4 (55-61)
i. " " " +5 (62-68)
j. " " " +6 (69-75)
k. Total Man Years of Effort (76-78)

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
					1			2							
27	28	29	30	31	32	33	34	35	36	37	38	39	40		
41	42	43	44	45	46	47	48	49	50	51	52	53	54		
55	56	57	58	59	60	61	62	63	64	65	66	67	68		
69	70	71	72	73	74	75	76	77	78						

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

- (A) Mertz, W., PhD., Dept of Biological Chemistry,
WRAIR, WRAMC, Washington, D. C., 20012, 576-3527
or Interdepartmental Code 198, Ext 3527 49
- (A) Harlow, D. R., M.S., Dept of Medical Zoology, Div
of Comm Dis & Immunol, WRAIR, WRAMC, Washington,
D. C., 20012, 576-3361 or Interdepartmental Code
198, Ext 3361 49
- (A) Williams, J. S., Dept of Medical Zoology, Div of
Comm Dis & Immunol, WRAIR, WRAMC, Washington, D.C.,
20012, 576-3055 or Interdepartmental Code 198,
Ext 3055 49

REPORTS. Annual Progress Report, Walter Reed Army Institute of
Research, 1 July 1963 - 30 June 1964.

ANNUAL PROGRESS REPORT

Project No. 3A013001A814 Title: Communicable Diseases and Immunology

Task No. 01 Title: Communicable diseases

Subtask No. 21 Title: Spirometra mansonoides infection and glucose metabolism

Description: Sparganosis in man is caused by the larval (sparganum) stage of species of broad tapeworm of the genus Spirometra. These larvae normally develop in frogs, snakes or amphibious mammals, but when the opportunity is afforded they can live in man and other terrestrial mammals. In man the largest number of infections have been reported from southeast Asia, Japan and Korea, but scattered cases are known from almost every part of the world. Recently Mueller reported that mice infected with spargana of Spirometra mansonoides showed accelerated weight gains compared with carefully matched controls. The gain could not be accounted for by the weight of the parasite or associated tissue reactions. The infected animals were simply larger and heavier due to abnormal accumulation of fat. A series of investigations was begun to gain some insight on, a) the possible role which parasites may play in influencing the growth and weight of their hosts; b) the factor(s) responsible for the fat accumulation in the infected animals; and c) the possibility of establishing a model for studies on adiposity and metabolic abnormalities.

Progress:

Effects of Spirometra mansonoides infection on carbohydrate metabolism. I. Glucose tolerance rates in mice.

The glucose tolerance test is a well established diagnostic procedure in clinical medicine. Although this test has been used for over 40 years in man, it is regarded as impractical for small laboratory animals such as mice because it requires relatively large samples of blood collected at close intervals. This limitation has prevented the utilization of this diagnostic procedure in most parasitic infections for which the mouse is the most suitable laboratory animal. A new method of biochemical analysis, permitting accurate measurements on a few microliters of blood, has made possible the development of a glucose tolerance test for mice. As indicated in Table I, the reproducibility of determinations made on deproteinized blood samples collected by calibrated polyethylene micropipettes was excellent. Similarly, preliminary studies indicated that optimal results

Table I

The reproducibility of duplicate blood glucose samples in fasted mice

Mouse #	Blood glucose levels in mg%	
	1st Sample	2nd Sample
1	65	65
2	75	76
4	77	72
5	62	64
6	65	62
7	79	81
8	59	61
9	94	93
10	92	91
11	65	69
12	79	81
13	79	79
14	53	53
15	92	105
17	90	90
18	102	101
20	66	67
21	111	113
22	90	90
23	85	84
24	69	68
25	71	69
Mean	69	69

could be obtained when 1 mg of glucose per gram of body weight was introduced intravenously and when the removal rate was observed at 15 minute intervals for 45 to 60 minutes. Per cent removal rates were calculated as the slope of the linear regression line of the logarithm of the mean excess glucose versus time expressed in minutes.

A series of experiments was conducted to examine some of the variables which may alter the glucose removal rates. In the first series, female mice of three different strains and of weight groups varying from very light to heavy (less than 15 to over 25 grams) were compared. As indicated in Tables II and III no significant differences were observed in the glucose removal rates of different weight groups. Conversely, when mice of the strain "BALBC" were studied (Table IV) there was conclusive evidence of a statistically significant difference between the two smaller groups and the group weighing more than 24.9 grams. When the removal rate of these three strains was compared, it appeared that mice of the "Cinnamon" and "CHR" strains are very nearly alike. Conversely, the mice of BALBC showed quite a highly significant difference from that of the other two species. In general, their removal rates were significantly higher.

A second series of experiments was conducted to compare in the mice of the BALBC strain the removal rates of both sexes (Tables IV, V, VI). Although some differences were observed, they were not statistically significant. In all experiments, per cent removal rates were calculated at 45 and at 60 minutes after introduction of the intravenous glucose. When the linear regression lines were calculated and comparisons were made over the range of time it was found that a better fit was obtained at 45 minutes than at 60. On all the graphs the range 15 to 45 minutes was quite linear, but in several of them the 60-minute point was elevated. This was particularly so with the BALBC strain which had a faster removal rate and in which a significant difference was observed whether the per cent removal rate was calculated at 45 or 60 minutes.

It was concluded that studies on the glucose removal rates in mice are feasible and reliable, that mice of the BALBC strain had consistently a higher removal rate than those of the other two strains tested, that no consistent significant differences are associated with different weight groups and that the estimate of the removal rate could be more precisely done at 45 minutes. On the basis of these results, comparisons were made of the glucose removal rate in female BALBC mice at various intervals following infection with Spirometra mansonoides. The influence of this infection in altering the glucose removal rates of its host is being observed at varying intervals from the time of infection. This study is still in progress.

Table II
The glucose removal rates in female mice of the "Cinnamon" strain according to weight groups

Weight Group (9R)	No. of Mice	Mean Weight	Mean Fasting Glucose Level	Average Excess Glucose			Per cent Removal Rate (45 min)	Per cent Removal Rate (60 min)
				15 min	30 min	45 min		
15.0	9	12.8	84	175	127	96	2.00	1.70
15.1-19.9	10	18.9	68	135	92	72	2.09	2.03
20.0-24.9	14	21.5	68	133	95	70	2.14	1.97
24.9	12	26.2	83	207	164	131	1.53	1.34
All weight groups	45	20.4	75.2	161	120	94	1.79	1.64
All except 24.9	33	18.3	72.5	145	103	80	1.98	1.89

Table III
The glucose removal rates in female mice of the "CHR" strain according to weight groups

Weight Group (9R)	No. of Mice	Mean Weight	Mean Fasting Glucose Level	Average Excess Glucose				Per cent Removal Rate (45 min)	Per cent Removal Rate (60 min)
				15 min	30 min	45 min	60 min		
15.0	9	13.7	75	161	123	86	62	2.09	2.14
15.1-19.9	24	18.7	73	142	100	82	62	1.83	1.79
20.0-24.9	18	22.3	83	135	93	72	55	2.09	1.97
24.9	15	26.3	88	143	113	91	77	1.51	1.38
All weight groups	66	20.7	79.2	143	108	83	63	1.81	1.81
All except 24.9	51	19.1	76.8	143	106	81	59	1.89	1.96

Table IV
The glucose removal rates in female mice of the BALBC strain according to weight groups

Weight Group (9R)	No. of Mice	Mean Weight	Mean Fasting Glucose Level	Average Excess Glucose			Per cent Removal Rate (45 min)	Per cent Removal Rate (60 min)
				15 min	30 min	45 min		
15.0	12	14.2	65	197	108	82	2.89	2.43
15.1-19.9	22	17.4	68	141	82	55	3.14	2.42
20.0-24.9	10	21.3	83	122	70	54	2.72	1.36
24.9	7	25.6	119	121	88	61	2.28	1.97
All weight groups	51	18.5	77	148	88	62	2.90	2.21
All except 24.9	44	17.4	70.3	152	88	62	2.98	2.27

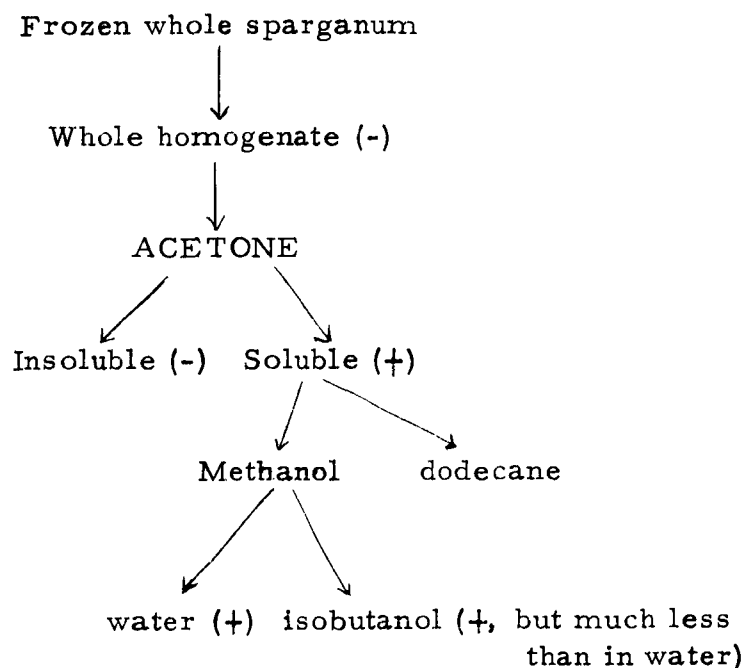
Table V
The glucose removal rates in male mice of the BALBC strain according to weight groups

Weight Group (9R)	No. of Mice	Mean Weight	Mean Fasting Glucose Level	Average Excess Glucose			Per cent Removal Rate	
				15 min	30 min	45 min	60 min	(60 min)
15.1-19.9	10	16.9	85	147	109	81	72	1.99
20.0-24.9	15	22.8	72	137	85	61	50	2.7
24.9	10	26.5	86	138	90	57	42	2.95
All weight groups	35	22.0	81.0	140.7	94.7	66.3	54.7	2.54
								2.25

Table VI
The glucose removal rates in male and female mice of the BALBC strain
according to weight groups

Weight Group (9R)	No. of Mice	Mean Weight	Mean Fasting Glucose Level	Average Excess Glucose			Per cent Removal Rate (45 min)	Per cent Removal Rate (60 min)
				15 min	30 min	45 min		
15.0								
15.1-20.0	32	17.3	73	143	91	63	58	2.73
								2.05
20.0-24.9	25	22.2	76	131	79	58	50	2.72
								2.13
24.9	17	26.1	99	131	89	59	46	2.66
								2.37
All weight groups	74	20.9	79.9	135	86.3	60.0	51.3	2.70
								2.18

Table VII
Flow Chart of Extracting Procedure



(+) Activity present
(-) No activity

Table VIII
In vitro assay results for fractions listed on Table XXVI

Level of Fractionation on Table XXVI			
Fraction	Acetone Soluble	Water	Isobutanol
Activity (Percentage increase over controls)	82	106	8
Dry weight (Per effective dose)	375 µgm	190 µgm	Not done
Purification	1:133	1:263	Not done

An insulin-like activity from the sparganum. After it was reported that the growth rates of mice are stimulated by infection with spargana of Spirometra mansonoides, studies were initiated in cooperation with the Division of Biochemistry to determine the biochemical mechanism of this phenomenon. It was observed in preliminary experiments that epididymal adipose tissue and diaphragm obtained from infected mice oxidized glucose- C^{14} to $C^{14}O_2$ in vitro at 2-3 times the rate of controls. A similar stimulation of glucose oxidation was observed when acetone extracts of frozen S. mansonoides spargana were added in vitro to epididymal fat tissue of rats. Using this assay to test various fractions, an extracting procedure was established (Table VII). Several other extracting procedures such as heptane against water, ethanol against decane, acetone against dodecane were tried without favorable results. Similarly, negative results were obtained with charcoal filtration.

As can be seen in Table VIII, the water soluble fraction obtained from the three partitioning steps can double the glucose oxidation rate in vitro. This fraction, obtained from 50 milligram of frozen sparganum, weighed only 200 micrograms and represents a 1:250 purification of the starting material.

Further modifications of the extracting procedure will be performed until the fraction can be subjected to various chemical and biological tests; the final objective being chemical identification and quantitation of biological activity.

Summary and Conclusions:

Infection in mice with the sparganum of Spirometra mansonoides produces abnormal increases in weight. A glucose tolerance test for mice was developed to determine the influence of this infection on the carbohydrate metabolism of the host. Experiments were conducted to examine some of the variables which may alter the glucose removal rates and to study the influence of this infection in altering the glucose removal rate in mice at regular intervals. Attempts to determine the biochemical mechanism of this phenomenon revealed that extracts of spargana produced a stimulation of glucose oxidation. Greatest activity was found in the water soluble fraction of the worms after extraction with acetone and methanol.

ARMY RESEARCH TASK REPORT			REPORTS CONTROL SYMBOL CSCRD-6(R2)
ACCESSION NUMBER 36191	PROJECT, TASK, OR SUBTASK NO. 3A025601A8060101		
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D. C., 20315	2. FUNDING AGENCY Army Medical R&D Command Office of The Surgeon General Washington, D. C., 20315		
3. CONTRACTING AGENCY NA	4. CONTRACTOR AND/OR GOV'T LABORATORY A Walter Reed Army Inst of Rsch Walter Reed Army Medical Center Washington, D. C., 20012 723-1000, Ext 3552		
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Gould, Douglas J., Ph.D., Department of Entomology, Division of Comm. Dis. & Immun., WRAIR, WRAMC, Washington, D. C., 20012 576-3719 or Interdepartmental Code 198, Ext 3719 See Continuation Sheet			
6. TITLE OF: PROJECT <input type="checkbox"/> TASK <input type="checkbox"/> SUBTASK <input checked="" type="checkbox"/> Ecology and control of disease vectors and reservoirs (U)			
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964			
8. RESUME (U) Experimental studies on the transmission of arthropod-borne diseases of military significance are carried on under this subtask. The principal objective of these studies is the incrimination of the arthropod vectors of malarial parasites and of arboviruses. Efforts are concentrated on the evaluation of the effects of intrinsic and extrinsic (temperature, relative humidity, photoperiod, etc.) factors on the vector efficiency of these arthropods. The mechanics of the infection process and the pattern of multiplication of pathogens in mosquitoes are being studied in an analysis of the vectorability of these arthropods. The potential role of arthropods and vertebrates in the ecology of arboviruses is being investigated through studies on the significance of birds and mammals as sources of infection for mosquitoes and on the host preferences of potential vector species. In an effort to develop experimental models for the investigation of malarial infections, attempts are being undertaken to adapt malarial parasites to new vertebrate and mosquito hosts. The ecology, bionomics and behavior of culicine and anopheline mosquitoes are being studied both in the field and laboratory with the objective of eventual laboratory colonization of vector species for experimental purposes. Procedures for mass rearing of anophelines are expected to proceed from these studies.			
9. KEY WORDS Medical entomology, malaria, dengue, viral encephalitides, sandfly fevers, vector incrimination, mosquito ecology, disease transmission.			
10. SUPPORTING PROJECTS Not applicable			
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet	12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet		
<div style="display: flex; justify-content: space-between;"> DA FORM 1309R 1 June 63 PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of ____ </div>			

ACCESSION NO.

36191

ARMY RESEARCH TASK REPORT

13. PROJECT, TASK OR
SUBTASK NUMBER

11	22	23 24	25 26	27 28	29
3 A 0 2 5 6 0 1 A 8 0 6	0 1	0 1			

14. DATE OF REPORT (30-33)

30	33	34
0 6 6 4	4	

15. SECURITY OF WORK (34)

16. TYPE OF REPORT

35	36	47 48 49 50 51	52	55
1				

17. SCIENTIFIC FIELD

 a. Topical Classific. (56-61)
 b. Functional Class (62-64)

56	61	62	64
0 1 0 6 0 8			

18. OSD CLASSIFICATION

(65-66)

19. R&D CATEGORY (67)

65 66	67
A R	2

20. CONTRACT NUMBER

11 12	13 14	15	17	18	21	22	26	27
D A								

21. GRANT NUMBER

28 29	30	33	34 35	36	38	39 40	41	45	46
D A							G		

22. ESTIMATED COMPLET.
DATES

47	51	52	56	57	61	62	66	67	71
1 C O N T	2			3		4		5	

23. PRIORITY (11-14)

24. PROGRAM ELEMENT
(15-26)

11	14	15	26
	1	6 • 2 1 • 5 6 • 0 1 • 1	

25. CMR&D CODES

27	29	30	32	33	35
N / A					

26. CDOG REFERENCE

a. Paragraph No. (36-44)

b. Functional Group (45)

36	39	40	41 42	43 44	45
					6

27. FUNDING

a. Est. Total Cost (11-15)

b. % Spent Intern. (16-18)

" " Extern. (19-21)

c. Total Obligation (22-26)

d. Progmd. Cur. FY (27-33)

e. " " " +1 (34-40)

f. " " " +2 (41-47)

g. " " " +3 (48-54)

h. " " " +4 (55-61)

i. " " " +5 (62-68)

j. " " " +6 (69-75)

k. Total Man Years of
Effort (76-78)

11	15	16	18	19	21	22	26
		1		2			
27 28	29	33	34 35	36	40		
41 42	43	47	48 49	50	54		
55 56	57	61	62 63	64	68		
69 70	71	75	76	78			

DA FORM 1309R
1 June 63

Previous Editions are Obsolete

Page 2 of _____

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

- (A) Atwood, E. L., B. S., Division of Preventive Medicine, WRAIR
WRAMC, Washington, D. C., 20012
576-2480 or Interdepartmental Code 198, Ext 2480 49
- (A) Buescher, E. L., Lt Col, MC, Dept of Virus Diseases, Div of
Communicable Disease and Immunology, WRAIR, WRAMC,
Washington, D. C., 20012
576-3757 or Interdepartmental Code 198, Ext 3757 49
- (A) Collins, M. J., M. S., Dept of Veterinary Science, University
of Maryland, College Park, Md. 20742-9800, Ext 697 18
- (A) Hayes, D. E., Dept of Entomology, Div of Communicable
Disease and Immunology, WRAIR, WRAMC, Washington, D. C., 20012
576-3724 or Interdepartmental Code 198, Ext 3724 49
- (A) Ludlam, K. W., B. S., Dept of Entomology, University of
Maryland, College Park, Md. 20742-9800, Ext 317 18
- (A) Moussa, M. A., Capt, MSC, Dept of Entomology, Div of
Communicable Disease and Immunology, WRAIR, WRAMC,
Washington, D. C., 20012
576-3724 or Interdepartmental Code 198, Ext 3724 49
- (A) Scheider, F. G., Capt, MC, Dept of Virus Diseases, Div of
Communicable Disease and Immunology, WRAIR, WRAMC,
Washington, D. C., 20012
576-3757 or Interdepartmental Code 198, Ext 3757 49
- (A) Suyemoto, W., B. S., Dept of Entomology, Div of Communicable
Disease and Immunology, WRAIR, WRAMC, Washington, D. C., 20012
576-2553 or Interdepartmental Code 198, Ext 2553 49
- (A) Ward, R. A., Ph.D., Dept of Entomology, Div of Communicable
Disease and Immunology, WRAIR, WRAMC, Washington, D. C., 20012
576-2087 or Interdepartmental Code 198, Ext 2087 49

REPORTS. Annual Progress Report, Walter Reed Army Institute of
Research, 1 July 1963 - 30 June 1964.

Eldridge, B. F.: The influence of daily photoperiod on blood-
feeding activity of Culex tritaeniorhynchus Giles. Amer. Jour.
of Hyg., 77:49-53, 1963.

Eldridge, B. F., Scanlon, J. E. and Orenstein, E. M.: Notes on the
laboratory rearing of sand flies (Diptera: Psychodidae). Mosquito
News, 23:215-217, 1963.

REPORTS - Continued:

Thompson, E. G., Hayes, D. E. and Ludlam, K. W.: Notes on the feeding habits of Aedes sollicitans in the Chincoteague-Assateague Island area of Virginia. Mosquito News, 23:297-298, 1963.

Ward, R. A.,: Genetic aspects of the susceptibility of mosquitoes to malarial infection. Expt. Parasit., 13:328-341, 1963.

Ward, R. A. and Kitzmiller, J. B.: A list of laboratory colonies of Anopheline mosquitoes. Mosquito News, 23:291-297, 1963.

ANNUAL PROGRESS REPORT

Project No. 3A025601A806

Title: Military Preventive Medicine

Task No. 01

Title: Ecology and Control of Disease
Vectors and Reservoirs

Subtask No. 01

Title: Ecology and Control of Disease
Vectors and Reservoirs

Description: This task covers a variety of studies on the ecology and bionomics of arthropods in relation to several groups of pathogenic agents and to a variety of vertebrates involved in infectious disease ecology.

Progress:

1. Ecology of arboviruses on Assateague and Chincoteague Islands.

Studies on the ecology of mosquito-borne viruses in Assateague and Chincoteague islands were carried out during 1963 in continuation of the effort at identifying the arthropod and vertebrate hosts of EEE and the Bunyamwera group of viruses in that area. As in 1961 and 1962 there was no evidence of EEE virus dissemination on Assateague or Chincoteague; no isolations of this virus were obtained from over 25,000 mosquitoes collected there between 3 June and 28 October (Table I), and there was no evidence of encephalitis in human beings or equines in the area during this period. Some 654 sera were obtained from birds trapped between 1 May and 23 August, and four out of 283 of these sera which were tested for EEE virus antibodies by the neutralization technique were positive. The species involved (Brown thrasher, catbird, yellow-breasted chat and Black and White warbler) have all been serologically implicated in the EEE virus cycle during previous years. Neutralization tests run with sera from nine Assateague foals born in 1963 and with sera from 18 wild Sika deer (Cervus nippon) trapped on Assateague during January and February, 1964 were negative for EEE antibodies. However, 31 of 52 Assateague and Chincoteague ponies bled during the roundup in July had neutralizing antibodies for EEE virus while 22 of these sera were also positive for EEE antibodies by the HAI test. These animals were all adults of undetermined age and very probably were infected during the 1960 EEE epizootic on Assateague and Chincoteague. Ten of the Assateague Sika deer sera were also tested for hemagglutinin-inhibiting antibodies against a Bunyamwera-group virus (BeAr 7272) and four were positive at low titer (1:10-1:20).

While no EEE virus was recovered from mosquitoes collected in 1963, two viruses were isolated in suckling mice from Aedes taeniorhynchus and a third was recovered from A. sollicitans (Table I). All three positive pools were collected on 21 August from a light trap located in the woods on Assateague. The 1963 isolates (designated M1724, M1727 and M1728), like four of the seven viruses recovered in 1961, are related to the Bunyamwera group of arboviruses by their complement-fixing reactivity (Table II). On the other hand, none of the four 1962 isolates have shown evidence of relation to the Bunyamwera group nor have they reacted by complement-fixation with antisera prepared against WEE, SLE, Dengue III, Anopheles A, Sicilian and Naples SFF viruses. The 1963 isolates are chloroform-sensitive as were the 1961 and 1962 isolates.

Table I

Virus isolations from mosquitoes collected on Assateague
and Chincoteague Islands during 1963

Species	Total specimens	No. pools tested	No. pools positive
<u>Aedes canadensis</u>	16	1	0
<u>A. cantator</u>	725	23	0
<u>A. infirmatus</u>	40	2	0
<u>A. sollicitans</u>	20,651	327	1
<u>A. taeniorhynchus</u>	2,989	65	2
<u>Anopheles</u> <u>quadrimaculatus</u>	14	1	0
<u>Culex salinarius</u>	738	18	0
Total	25,173	437	3

Table II

Complement-fixing reactivity of Bunyamwera group
antisera to 1963 Assateague Isolates

Antiserum	Antigen			
	Homologous	M1724/ 63**	M1727/ 63**	M1728/ 63**
Batai	1:64	1:32	1:64	1:64
BeAr 7272	1:64	1:64	1:64	1:64
Bunyamwera	1:28	1:32	1:32	1:64
Guaroa	1:128	1:8	1:8	1:8
Ilesha	1:64	1:16	1:16	1:16
M291/61*	1:1024	1:128	1:128	1:128

*1961 Chincoteague isolate

**Antigen in excess

Between 1960 and 1962 less than a dozen specimen of Culiseta melanura were collected on Assateague and Chincoteague. This mosquito is believed to play an important role in the natural cycle of EEE virus, for more isolations of this virus have been recovered from wild caught C. melanura than from any other species in North America. Since C. melanura breeds in fresh water swamps intensive collecting was undertaken in 1963 in a swampy wooded area along the western shore of Assateague which seemed to be the most likely habitat on either island in which to find C. melanura. Five raccoon-baited traps, eight battery operated New Jersey light traps and four battery operated Chamberlain light traps were located in a random pattern through this area. The bait traps were operated continuously and mosquitoes collected from them three times weekly; the light traps were run twice weekly. The New Jersey traps were operated for a three-hour period immediately following sunset and the Chamberlain traps were run all night. The summer of 1963 was one of prolonged drought in this region so that insufficient water accumulated in these woods to permit much mosquito breeding. No collections of C. melanura were made in this area during 1963. Approximately 18,000 mosquitoes were collected in this woodland site, but the overwhelming majority of those collected were salt-marsh breeding species such as A. sollicitans and A. taeniorhynchus (Table III). This invasion of the woods site by salt-marsh mosquitoes is of particular interest for it provides an area of overlap where the exchange of arboviruses between woods and marsh mosquitoes could occur.

The estimated overall seasonal rates of infection of Aedes sollicitans, A. taeniorhynchus and Anopheles bradleyi populations with Bunyamwera group viruses in the Assateague-Chincoteague area during 1961 were respectively 3/10,000, 17/10,000 and 17/10,000. There was good evidence, however, of an increasing rate of infection in the A. sollicitans population by the end of the season, because the estimated rates for the periods 6 June - 28 July, 29 July - 24 August, 2 September - 6 October and 7 October - 11 November, respectively, were - 0/10,000, 2.6/10,000, 0.9/10,000 and 18/10,000 (seasonal average - 3.3/10,000).

In 1963 an effort was made to relate the virus infection rates in the salt-marsh mosquito populations to estimates of the size of these populations so as to provide a basis for assessing the degree of arbovirus dissemination by these mosquitoes. The total area of potential salt-marsh mosquito breeding sites on Chincoteague, the tidal marshes between Chincoteague and the mainland and on the Virginia half of Assateague was estimated by planimeter to be approximately 5,280 acres. This area was divided into eight primary sampling units varying in size from 187 to 883 acres that were roughly homogeneous as to elevation. Two secondary units .75 acres in size were randomly selected within each primary area. Each secondary unit was made up of 30 potential sampling plots. Within each secondary unit, two sampling plots each 9.5 feet by 114 feet 7 inches (.025 acres) were chosen at random. All 32 sampling plots were oriented in a north-south direction. At weekly intervals a standardized sweep-net collection was made from each of the sampling plots. The sweeping method appeared to be a useful tool in sampling adult marsh mosquito populations of three species - A. sollicitans, A. taeniorhynchus and A. cantator. Aedes sollicitans was the dominant species, comprising 99% of the total catch. Systematic application of this sampling method between August and October 1963 indicated that there were two

Table III

Mosquito fauna of the Woodland study area on Assateague Island,
Virginia as indicated by different sampling methods

Species	Percentage of total catch		
	Raccoon-baited trap	Chamberlain light trap	New Jersey light trap
<u>Aedes sollicitans</u>	49	53	68
<u>Aedes taeniorhynchus</u>	26	37	27
<u>Aedes cantator</u>	12	7	4
<u>Culex salinarius</u>	10	<1	<1
<u>Anopheles bradleyi</u>	2	1	<1
<u>Anopheles quadrimaculatus</u>	<1	<1	<1
<u>Aedes infirmatus</u>	<1		
<u>Aedes vexans</u>	<1	1	<1
<u>Culex territans</u>	<1		
<u>Psorophora ferox</u>	<1		
<u>Psorophora ciliata</u>		<1	
Total mosquito catch	8347	1712	7888

generations of A. sollicitans with peaks of activity during that period, one occurring early in August and the second late in September; a single brood of both A. taeniorhynchus and of A. cantator was observed with peaks of activity about mid-September and early October, respectively. Mosquito collections obtained by the sweeping method were applied to estimation of total A. sollicitans population sizes since the samples were collected from randomized unit areas of known size. These catches were expanded by the total acreage of the marsh area involved (Table IV). Estimates of total populations appeared to offer a useful measurement of variability between areas of comparable size especially in cases where density indices were too small for interpretation of variations. Total population estimates were not dependable for comparisons of the productivity of different areas of marsh, however. This was demonstrated when population estimates indicated the presence of more than a million adult A. sollicitans in marsh areas four, seven and eight despite the absence of any evidence of breeding sites on those areas. These observations reflected, instead, the invasion of those areas by mosquitoes from other sites. There was close agreement between the data obtained from the sweeping collections and those obtained from the trap-collections obtained from the Assateague woodland site. In the case of Aedes sollicitans, the collections from the bait traps and light-traps in the woods revealed the two peaks of activity occurring approximately two weeks later than their date of occurrence on the marshes as indicated by sweep net collections. Furthermore, an identical lag in the appearance of population peaks for A. taeniorhynchus and A. cantator in the woods was indicated by the trap collections. Finally, evidence of an earlier brood of A. taeniorhynchus in the first week of August was obtained from the trap collections although no such peak was observed in the levels of the marsh populations of A. taeniorhynchus. This dissimilarity may indicate either that the migratory flights of A. taeniorhynchus to the woods had occurred prior to the initiation of the sweep net collections or that they occurred over a short interval of time between weekly sweep net collections.

2. Effects of chemosterilants on malarial parasites.

It has been demonstrated that certain alkylating agents can interrupt the development of Plasmodium gallinaceum in Aedes aegypti and concurrently induce mosquito sterility without producing abnormal mortality. To determine the practicality of this new method of vector and parasite control for the control of human malaria, a trial laboratory experiment was conducted with the vivax-like malaria parasite, P. cynomolgi, of the rhesus monkey, Anopheles stephensi and the alkylating agent apholate ($C_{12}H_{24}N_9P_3$).

After receiving an infective blood meal, mosquitoes were transferred to pint jars which had the inner surface coated with apholate residues. They were kept in these jars for various intervals and then transferred to holding cages for observation. At day six an aliquot from each group was removed to make malarial oocyst counts. The results indicate that apholate caused a reduction in malarial oocyst development of considerable magnitude which appeared related to length of exposure (Table V). However, some oocysts did mature at these dosage levels and produced mature sporozoites. The mortality of treated mosquitoes was twice that of controls.

Table IV

Variations in populations of females Aedes sollicitans in marsh areas 1 - 4,
Chincoteague-Assateague Islands (1963)

Date	No. of mosquitoes (in thousands)/primary sampling area							
	1 (187 acres)		2 (745 acres)		3 (591 acres)		4 (732 acres)	
	Density per acre	Total population estimate	Density per acre	Total population estimate	Density per acre	Total population estimate	Density per acre	Total population estimate
August								
1 - 15	2.7	407	1.4	1,058	1.9	1,082	0.05	44
16 - 31	0.1	23	0.04	30	0.1	65	0.05	44
September								
1 - 15	0.04	6	0	0	0.01	6	0.02	15
16 - 30	20.4	3,809	5.6	4,178	8.4	4,938	0.7	498
October								
1 - 15	15.9	2,979	8	5,571	4.1	2,437	1.8	1,325
16 - 31	4.3	796	1.1	782	1.4	805	0.4	308

Table IV (con't.)

Variations in populations of females *Aedes sollicitans* in marsh areas 5 - 8,
Chincoteague-Assateague Islands (1963)

Date	No. of mosquitoes (in thousands)/primary sampling area							
	5 (635 acres)		6 (622 acres)		7 (854 acres)		8 (883 acres)	
	Density per acre	Total population estimate	Density per acre	Total population estimate	Density per acre	Total population estimate	Density per acre	Total population estimate
August								
1 - 15	0.3	159	0.9	566	0	0	0.02	18
16 - 31	0.5	273	0.3	168	0.02	17	0.1	97
September								
1 - 15	0.04	26	0.1	62	0	0	0	0
16 - 30	2.3	1,468	1.4	864	0.8	624	0.9	813
October								
1 - 15	5.2	3,292	7.1	4,371	0.3	231	0.48	424
16 - 31	0.8	470	3.7	2,294	0	0	0	0

Table V

Effect of apholate on Anopheles stephensi and Plasmodium cynomolgi

Apholate concentration	Length of exposure	Mean no. oocysts	Six day mortality
10 mg / ft ²	1 hr	52.1	24/50
10 mg / ft ²	2 hr	17.7	31/50
20 mg / ft ²	1 hr	201.6	25/50
20 mg / ft ²	2 hr	32.0	32/50
0 mg / ft ²	1 hr	325.8	13/50

3. Artificial membrane feeding of mosquitoes.

Studies of the nutrition, feeding behavior and vector potential of blood-sucking arthropods often call for a convenient, standardized technique for feeding defined meals in the laboratory. The membrane feeding technique is one approach to this problem.

A feeder-device was designed specifically for feeding mosquitoes on infectious agents through membranes. The feeder is in the form of a glass double-walled chamber. An inner chamber holds the feeding solution while warm water (38°C) circulates through the outer wall space. A membrane is placed on the bottom of the feeding chamber and the apparatus is placed over a mosquito cage. Various factors that influence the engorging responses of mosquitoes have been investigated.

Type of membrane. The feeding response of females of Aedes aegypti and Anopheles stephensi through four different membranes were compared. A. aegypti fed best through chick skins while A. stephensi fed well through Baudruche and parafilm membranes. Neither species fed through Saran Wrap. The Baudruche membrane was selected as a standard due to ease in handling and its ready acceptance by all species tested.

Type of food. When heparinized blood was provided to the mosquitoes in the membrane feeder, an average of 11 to 50 per cent of the mosquitoes fed, depending on the species (Table VI). Less than five per cent of the mosquitoes fed on solutions comprised of saline chick erythrocyte extract (CEE), 10% bovine serum-saline or 4% sucrose.

Phagostimulants. It has been shown that the addition of adenosine triphosphate (ATP) to nutritive solutions will increase the feeding response of mosquitoes. Various levels of this substance were added to CEE in two membranes (Table VII). to determine its optimal phagostimulant level. At a concentration of 0.001M, the lowest concentration tested, there was a demonstrable effect, but optimal results were obtained at a level at 0.005M. Consequently, this level was adopted in all later feeding experiments. Addition of the mosquito attractants lysine and alanine to feeding solutions did not stimulate membrane feeding by mosquito.

Comparison of species. The responses of six mosquito species to various feeding solutions are indicated in Table VI. Of the six species, only Aedes aegypti fed more profusely on chicken blood than on solutions with ATP added. In general all species tested, with the exception of Culex pipiens, fed well through the Baudruche membrane.

4. Viral isolates from sandflies.

The characterization and identification of viral agents isolated from sandflies from Iran and Pakistan has continued. Strain IS-92, isolated from Sergentomyia females in June of 1959 at Said Pur, Pakistan has been identified as a member of prototype IP-58. In the characterization of these sandfly isolates, hyperimmune ascitic fluid prepared against four of the prototype viruses isolated in Iran and Pakistan (Naples, Sicilian, IP-81, and IP-58) were used in the neutralization tests in suckling and weanling mice. Prototype IP-47 did not produce neutralizing antibody in mouse ascitic fluid. The characterization of further isolates from Sergentomyia females and Phlebotomus males is continuing and preparation of HA antigens from these agents is underway.

Summary and Conclusions:

1. No evidence was obtained for the dissemination of EEE virus on Assateague and Chincoteague islands during 1963. Two isolations of arboviruses were recovered from Aedes taeniorhynchus and a third from Aedes sollicitans. These isolates appear to belong to the Bunyamwera group of viruses, as are the 1961 Assateague isolates, but are antigenically distinct from the 1962 isolates. Studies on the dynamics of the salt-marsh and woods mosquito populations in Assateague and Chincoteague islands were undertaken during 1963. A sampling method based upon randomized sweep-net collections was devised to determine fluctuations in size and density of salt-marsh mosquito populations in this area.

2. The effect of the chemosterilant apholate on malarial parasites was studied in Anopheles stephensi infected with Plasmodium cynomolgi. A marked reduction in oocyst count was observed but there was a 50 per cent increase in mosquito mortality.

3. A standardized membrane feeding technique was devised to evaluate membrane feeding by six species of mosquitoes belonging to the genera

Table VI

Percent of mosquito species feeding on solutions
through Baudruche membranes

Mosquito species	Feeding solution		
	chicken blood	CEE + 0.005M ATP	10% BS + 0.005M ATP
<u>Aedes aegypti</u>	47	39	38
<u>Aedes togoi</u>	54	54	64
<u>Anopheles stephensi</u>	37	72	51
<u>Armigeres subalbatus</u>	21	57	83
<u>Culex tritaeniorhynchus</u>	17	19	29
<u>Culex pipiens pallens</u>	11	9	14

Table VII

Percent of Aedes aegypti feeding on CEE through
membranes at various levels of ATP

Type of membrane	Concentration of ATP (Moles)					
	0.000	0.001	0.002	0.005	0.010	0.020
Chick skin	4	51	87	92	79	77
Baudruche	2	19	51	59	59	20

Aedes, Culex, Anopheles and Armigeres. The addition of ATP to feeding solutions tested was found to have a phagostimulatory effect on these species. Use of this technique to quantitate the feeding of infectious agents to mosquitoes is planned.

4. Isolate IS-92, recovered from sandflies of the genus Sergentomyia collected in Pakistan, has been identified as belonging to the prototype virus IP-58 by neutralization test.

ARMY RESEARCH TASK REPORT				REPORTS CONTROL SYMBOL CSCRD-6(R2)	
ACCESSION NUMBER 36192			PROJECT, TASK, OR SUBTASK NO. 3A025601A8060201		
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D.C., 20315			2. FUNDING AGENCY Army Medical R&D Command Office of The Surgeon General Washington, D.C., 20315		
3. CONTRACTING AGENCY NA			4. CONTRACTOR AND/OR GOV'T LABORATORY A Walter Reed Army Inst of Rsch Walter Reed Army Medical Center Washington, D.C., 20012 723-1000, Ext 3552		
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Gauld, Ross L., M.D., Dr. P.H., Director, Division of Preventive Medicine, WRAIR, WRAMC, Washington, D.C., 20012 576-3553 or Interdepartmental Code 198, Ext 3553 See Continuation Sheet 49					
6. TITLE OF: PROJECT <input type="checkbox"/> TASK <input type="checkbox"/> SUBTASK <input checked="" type="checkbox"/> Global health data (u)					
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964					
8. RESUME (U) Preparation and Publication of Health Data Reports. These are unclassified reports of health and sanitary conditions in foreign countries for the use of Army Medical Service officers. They describe the geography, climate, religion, living conditions, animals and plants of medical importance, water supply, methods of waste and sewage disposal, diseases present, medical facilities, etc., of each country reported on. Material is obtained from the published literature (medical and non-medical), from briefings of persons familiar with the country and sometimes by visits to the area.					
9. KEY WORDS Public health, epidemiology, communicable diseases, disease vectors, sanitary engineering, geography, climatology.					
10. SUPPORTING PROJECTS Not Applicable					
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet			12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet		
<div style="display: flex; justify-content: space-between;"> DA FORM 1309R 1 June 63 PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of _____ </div>					

ACCESSION NO. 36192		ARMY RESEARCH TASK REPORT	
13. PROJECT, TASK OR SUBTASK NUMBER	11	22	23 24 25 26 27 28 29
	3	A 0 2 5 6 0 1 A 8 0 6	0 2 0 1
	14. DATE OF REPORT (30-33)	30	33
	15. SECURITY OF WORK (34)	0 6 6 4	4
	16. TYPE OF REPORT	35	36
17. SCIENTIFIC FIELD a. Topical Classific. (56-61) b. Functional Class (62-64)	56	61	62 64
	0 1 0 6 0 8		
18. OSD CLASSIFICATION (65-66)	65 66	67	
19. R&D CATEGORY (67)	A R	2	
20. CONTRACT NUMBER	11 12	13 14	15 17 18 21 22 26 27
21. GRANT NUMBER	28 29	30	33 34 35 36 38 39 40 41 45 46
	D A		
22. ESTIMATED COMPLET. DATES	47	51	52 56 57 61 62 66 67 71
23. PRIORITY (11-14) 24. PROGRAM ELEMENT (15-26)	11	14	15 26
		1	6 • 2 1 • 5 6 • 0 1 • 1
25. CMR&D CODES	27	29	30 32 33 35
26. CDOG REFERENCE a. Paragraph No. (36-44) b. Functional Group (45)	36	39	40 41 42 43 44 45
			6
27. FUNDING a. Est. Total Cost (11-15) b. % Spent Intern. (16-18) " " Extern. (19-21) c. Total Obligation (22-26) d. Progrmd. Cur. FY (27-33) e. " " " +1 (34-40) f. " " " +2 (41-47) g. " " " +3 (48-54) h. " " " +4 (55-61) i. " " " +5 (62-68) j. " " " +6 (69-75) k. Total Man Years of Effort (76-78)	11	15	16 18 19 21 22 26
			1
	27 28	29	33 34 35 36 40
	41 42	43	47 48 49 50 54
	55 56	57	61 62 63 64 68
	69 70	71	75 76 78

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

(A) Bowditch, S.J., M.D., Dept. of Health Data,
Div of Prev Med, WRAIR, WRAMC, Washington, D.C. 20012
576-2061 or Interdepartmental Code 198, Ext 2061

49

REPORTS. Annual Progress Report, Walter Reed Army Institute
of Research, 1 July 1963 - 30 June 1964.

ANNUAL PROGRESS REPORT

Project No. 3A025601A806

Title: Military Preventive Medicine

Task No. 02

Title: Global Health Data

Subtask No. 01

Title: Global Health Data

Description: Health Data Reports are prepared for the use of Army Medical Service officers and contain unclassified information regarding the health and sanitary conditions likely to be encountered in foreign countries to which they are deployed.

Progress: Health Data Reports are unclassified reports of health and sanitary conditions in foreign countries for the use of Army Medical Service officers. They describe the geography, climate, religion, living conditions, animals and plants of medical importance, water supply, methods of waste and sewage disposal, diseases present, medical facilities, etc., of each country reported on.

To the end of FY 1964, 25 countries had been reported on as follows: Panama, Cambodia, Cuba, Liberia, Kenya, Haiti, Dominican Republic, South Viet Nam, Thailand, Nepal, Iraq, Laos, El Salvador, Ethiopia, Iran, British Guiana, Federation of Rhodesia and Nyasaland, Equador, Yemen, Tanganyika, Honduras, Ghana, Nigeria, Angola, Cyprus. The last 5 of these were published during the past year. In addition, reports on the following countries are in varying stages moving toward completion: Venezuela, Congo, Panama (revision), Brazil, Senegal, Mozambique, Burma, Indonesia and India.

ARMY RESEARCH TASK REPORT				REPORTS CONTROL SYMBOL CSCRD-4(R2)	
ACCESSION NUMBER 36193			PROJECT, TASK, OR SUBTASK NO. 3A025601A8110101		
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D. C. 20315			2. FUNDING AGENCY Army Medical R&D Command Office of The Surgeon General Washington, D. C. 20315		
3. CONTRACTING AGENCY NA			4. CONTRACTOR AND/OR GOV'T LABORATORY A Walter Reed Army Inst of Rsch Walter Reed Army Medical Center Washington, D. C. 20012 723-1000, Ext 3552		
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Hansen, James L., Col, MC, Director, US Army-SEATO Medical Research Laboratory, Bangkok, Thailand <div style="text-align: right;">See Continuation Sheet</div>					
6. TITLE OF: PROJECT <input type="checkbox"/> TASK <input type="checkbox"/> U. S. Military Medical Research Program, SUBTASK <input checked="" type="checkbox"/> SEASIA (Thailand) (U)					
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964					
8. RESUME' (U)As a part of the overall area study of Thailand, detailed investigations have been made of the epidemiology and clinical aspects of Thai hemorrhagic fever and other arboviruses, of mosquitoes important as vectors of the foregoing and of malaria including colonization of <u>Anopheles balabacensis</u> , of the life cycle of <u>Opisthorchis viverrini</u> , of drug-resistant <u>Plasmodium falciparum</u> , of cholera and the other dysenteric diseases, of leptospirosis, and of a variety of non-infectious disease entities of military and civilian personnel.					
9. KEY WORDS Thailand, hemorrhagic fever, dengue, mosquito, malaria, cholera, leptospirosis.					
10. SUPPORTING PROJECTS Not Applicable					
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet			12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T.AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet		
DA FORM 1309R 1 June 63 PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of ____					

ACCESSION NO. 36193		ARMY RESEARCH TASK REPORT	
13. PROJECT, TASK OR SUBTASK NUMBER		11 22 23 24 25 26 27 28 29 3 A 0 2 5 6 0 1 A 8 1 1 0 1 0 1	
14. DATE OF REPORT (30-33)		30 33 34	
15. SECURITY OF WORK (34)		0 6 6 4 4	
16. TYPE OF REPORT		35 36 47 48 49 50 51 52 55	
17. SCIENTIFIC FIELD a. Topical Classific. (56-61) b. Functional Class (62-64)		56 61 62 64	
18. OSD CLASSIFICATION (65-66)		65 66 67	
19. R&D CATEGORY (67)		A R 2	
20. CONTRACT NUMBER		11 12 13 14 15 17 18 21 22 26 27	
21. GRANT NUMBER		28 29 30 33 34 35 36 38 39 40 41 45 46	
22. ESTIMATED COMPLET. DATES		47 51 52 56 57 61 62 66 67 7	
23. PRIORITY (11-14)		11 14 15 26	
24. PROGRAM ELEMENT (15-26)		6 • 2 1 • 5 6 • 0 1 • 1	
25. CMR&D CODES		27 29 30 32 33 35	
26. CDOG REFERENCE a. Paragraph No. (36-44) b. Functional Group (45)		36 39 40 41 42 43 44 45	
27. FUNDING		11 15 16 18 19 21 22 26 27 28 29 33 34 35 36 40 41 42 43 47 48 49 50 54 55 56 57 61 62 63 64 68 a. Est. Total Cost (11-15) b. % Spent Intern. (16-18) " " Extern. (19-21) c. Total Obligation (22-26) d. Programd. Cur. FY (27-33) e. " " " +1 (34-40) f. " " " +2 (41-47) g. " " " +3 (48-54) h. " " " +4 (55-61) i. " " " +5 (62-68) j. " " " +6 (69-75) k. Total Man Years of Effort (76-78)	

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

Inquiries concerning this program should be addressed to Director, WRAIR, WRAMC, Washington, D. C. 20012, 576-3551 or Interdepartmental Code 198, Ext 3551.

(A) Bourke, Anthony, Capt, MC, US Army-SEATO Medical Research Laboratory, Bangkok, Thailand	80
(A) Halstead, Scott B., Maj, MC, US Army-SEATO Medical Research Laboratory, Bangkok, Thailand	80
(A) Keefe, Thomas J., Capt, VC, US Army-SEATO Medical Research Laboratory, Bangkok, Thailand	80
(A) Marshall, Joe T., Jr., Ph.D., US Army-SEATO Medical Research Laboratory, Bangkok, Thailand	80
(A) Morris, John H., II, Lt Col, VC, US Army-SEATO Medical Research Laboratory, Bangkok, Thailand	80
(A) Noyes, Howard E., Ph.D., US Army-SEATO Medical Research Laboratory, Bangkok, Thailand	80
(A) Nye, Sylvanus W., Capt, USAF, MC, US Army-SEATO Medical Research Laboratory, Bangkok, Thailand	80
(A) Olsson, Ray A., Maj, MC, US Army-SEATO Medical Research Laboratory, Bangkok, Thailand	80
(A) Scanlon, John E., Maj, MSC, US Army-SEATO Medical Research Laboratory, Bangkok, Thailand	80
(A) Sobocinski, Philip Z., Capt, MSC, US Army-SEATO Medical Research Laboratory, Bangkok, Thailand	80
(A) Weinman, David, II, M.D., US Army-SEATO Medical Research Laboratory, Bangkok, Thailand	80
(A) Wykoff, Dale E., Maj, MSC, US Army-SEATO Medical Research Laboratory, Bangkok, Thailand	80

REPORTS. Annual Progress Report, SEATO Medical Research Laboratory and Clinical Research Center, Bangkok, Thailand, 1 April 1963 - 31 March 1964.

ARMY RESEARCH TASK REPORT		REPORTS CONTROL SYMBOL CSCRD-6(R2)
ACCESSION NUMBER 36194		PROJECT, TASK, OR SUBTASK NO. 3A025601A8110110
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D. C. 20315	2. FUNDING AGENCY Army Medical R&D Command Office of The Surgeon General Washington, D. C. 20315	
3. CONTRACTING AGENCY NA	4. CONTRACTOR AND/OR GOV'T LABORATORY A Walter Reed Army Inst of Rsch Walter Reed Army Medical Center Washington, D. C. 20012 723-1000, Ext 3552 49	
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Teschan, Paul E., Lt Col, MC, Chief, US Army Medical Research Team (WRAIR) Vietnam, Saigon, Vietnam 81		
6. TITLE OF: PROJECT <input type="checkbox"/> TASK <input type="checkbox"/> Military medical research program, SUBTASK <input checked="" type="checkbox"/> SEASIA (Vietnam) (U)		
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964		
8. RESUME (U) This subtask is reported separately.		
9. KEY WORDS Vietnam		
10. SUPPORTING PROJECTS Not Applicable		
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet		12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet
DA FORM 1300R 1 June 63		PAGE 1 of _____

ACCESSION NO.

36194

ARMY RESEARCH TASK REPORT

13. PROJECT, TASK OR
SUBTASK NUMBER

11	22	23 24	25 26	27 28	29
3 A 0 2 5 6 0 1 A 8 1 1		0 1	1 0		

14. DATE OF REPORT (30-33)

30 33 34

15. SECURITY OF WORK (34)

0 6 6 4 2

16. TYPE OF REPORT

35	36	47 48	49 50	51	52	55
1						

17. SCIENTIFIC FIELD

 a. Topical Classific. (56-61)
 b. Functional Class (62-64)

56	61	62	64
0 1 0 6 0 0			

18. OSD CLASSIFICATION

(65-66)

65 66
A R

19. R&D CATEGORY (67)

67
2

20. CONTRACT NUMBER

11 12	13 14	15	17	18	21	22	26	27
D A								

21. GRANT NUMBER

28 29	30	33	34 35	36	38	39 40	41	45	46
D A							G		

22. ESTIMATED COMPLET.
DATES

47	51	52	56	57	61	62	66	67	71
1 0 0 N T		2		3		4		5	

23. PRIORITY (11-14)

24. PROGRAM ELEMENT
(15-26)

11	14	15	26
	1	6 • 2 1 • 1 0 • 0 1 • 1	

25. CMR&D CODES

27	29	30	32	33	35
N	A				

26. CDOG REFERENCE

 a. Paragraph No. (36-44)
 b. Functional Group (45)

36	39	40	41 42	43 44	45
					6

27. FUNDING

 a. Est. Total Cost (11-15)
 b. % Spent Intern. (16-18)
 " " Extern. (19-21)
 c. Total Obligation (22-26)
 d. Progrmd. Cur. FY (27-33)
 e. " " " +1 (34-40)
 f. " " " +2 (41-47)
 g. " " " +3 (48-54)
 h. " " " +4 (55-61)
 i. " " " +5 (62-68)
 j. " " " +6 (69-75)
 k. Total Man Years of
 Effort (76-78)

11	15	16	18	19	21	22	26
		1		2			
27 28	29	33	34 35	36	40		
41 42	43	47	48 49	50	54		
55 56	57	61	62 63	64	68		
69 70	71	75	76	78			

ARMY RESEARCH TASK REPORT		REPORTS CONTROL SYMBOL CSCRD-4(R2)
ACCESSION NUMBER 36195	PROJECT, TASK, OR SUBTASK NO. 3A025601A8110115	
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D. C. 20315	2. FUNDING AGENCY Army Medical R&D Command Office of The Surgeon General Washington, D. C. 20315	
3. CONTRACTING AGENCY NA	4. CONTRACTOR AND/OR GOV'T LABORATORY A Walter Reed Army Inst of Rsch Walter Reed Army Medical Center Washington, D. C. 20012 723-1000, Ext 3552	
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Baker, Hinton J., Lt Col, MC, WRAIR, WRAMC, Washington, D. C. 20012, 576-3518 or Interdepartmental Code 198, Ext 3518		
6. TITLE OF: PROJECT <input type="checkbox"/> TASK <input type="checkbox"/> SUBTASK <input checked="" type="checkbox"/> Military medical research program, SEASIA (Malaysia)		
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964		
8. RESUME (U) This is a carrier subtask to provide for WRAIR personnel stationed in Malaysia working in association with the U. S. Army Medical Unit, Kuala Lumpur, Malaya. Scientific data is reported by that unit (see Grant No. 416-121, Accession No. 3137926).		
9. KEY WORDS Malaysia		
10. SUPPORTING PROJECTS Not Applicable		
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet	12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet	
<div style="display: flex; justify-content: space-between;"> DA FORM 1309R 1 June 63 PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of ____ </div>		

ACCESSION NO.		ARMY RESEARCH TASK REPORT	
36195			
13. PROJECT, TASK OR SUBTASK NUMBER	11 22 23 24 25 26 27 28 29 3 A 0 2 5 6 0 1 A 8 1 1 0 1 1 5		
14. DATE OF REPORT (30-33)	30 33 34 0 6 6 4 4		
15. SECURITY OF WORK (34)			
16. TYPE OF REPORT	35 36 47 48 49 50 51 52 55 1		
17. SCIENTIFIC FIELD a. Topical Classific. (56-61) b. Functional Class (62-64)	56 61 62 64 0 1 0 6 0 0		
18. OSD CLASSIFICATION (65-66)	65 66 67 A R 2		
19. R&D CATEGORY (67)			
20. CONTRACT NUMBER	11 12 13 14 15 17 18 21 22 26 27 D A		
21. GRANT NUMBER	28 29 30 33 34 35 36 38 39 40 41 45 46 D A G		
22. ESTIMATED COMPLET. DATES	47 51 52 56 57 61 62 66 67 71 1 C O N T 2 3 4 5		
23. PRIORITY (11-14)	11 14 15 26 1 6 • 2 1 • 5 6 • 0 1 • 1		
24. PROGRAM ELEMENT (15-26)			
25. CMR&D CODES	27 29 30 32 33 35 N / A		
26. CDOG REFERENCE a. Paragraph No. (36-44) b. Functional Group (45)	36 39 40 41 42 43 44 45 6		
27. FUNDING a. Est. Total Cost (11-15) b. % Spent Intern. (16-18) " " Extern. (19-21) c. Total Obligation (22-26) d. Progmd. Cor. FY (27-33) e. " " " +1 (34-40) f. " " " +2 (41-47) g. " " " +3 (48-54) h. " " " +4 (55-61) i. " " " +5 (62-68) j. " " " +6 (69-75) k. Total Man Years of Effort (76-78)	11 15 16 18 19 21 22 26 1 2 27 28 29 33 34 35 36 40 41 42 43 47 48 49 50 54 55 56 57 61 62 63 64 68 69 70 71 75 76 78		

ARMY RESEARCH TASK REPORT				REPORTS CONTROL SYMBOL CSCRD-6(R2)	
ACCESSION NUMBER 36196			PROJECT, TASK, OR SUBTASK NO. 3A025601A8110121		
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D. C., 20315			2. FUNDING AGENCY Army Medical R&D Command Office of The Surgeon General Washington, D. C., 20315		
3. CONTRACTING AGENCY NA			4. CONTRACTOR AND/OR GOV'T LABORATORY A Walter Reed Army Inst of Rsch Walter Reed Army Medical Center Washington, D. C. 20012 723-1000, Ext 3552		
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Sadun, Elvio H., Sc.D., Dept of Medical Zoology, Div of Communicable Disease and Immunology, WRAIR, WRAMC, Washington, D. C., 20012 576-3308 or Interdepartmental Code 198, Ext 3308; See Continuation Sheet 49					
6. TITLE OF: PROJECT <input type="checkbox"/> TASK <input type="checkbox"/> SUBTASK <input checked="" type="checkbox"/> Military medical research program, SEASIA. (WRAIR) (U)					
7. DATE OF REPORT DAY MONTH YEAR					
8. RESUME (U) A modified fluorescent antibody test for diagnosis of human malaria was developed and evaluated. <u>Plasmodium berghei</u> studies were concerned with physiological pathology in mice, development of a direct parasite enumeration method, immunodiffusion analysis, LD ₅₀ for mice, search for suitable hosts, immunizing effect of irradiated parasites in mice. Rickettsial disease studies indicate widespread endemicity of scrub typhus, Q fever and a member of the spotted fever group throughout the various physiographic provinces of continental Thailand. Serological evidence of prior infection with spotted fever and typhus group rickettsiae, scrub typhus and Q fever was found in human sera from central South Vietnam. Plague studies were concerned with ecology in South Vietnam and development and testing of a new transport medium. Bacteriologic characterization of traumatic wounds from 131 patients wounded in Vietnam was accomplished. Leptospirosis studies were conducted to elucidate epidemiologic and serologic factors bearing on the occurrence of loci of infections, to characterize types of leptospiras and their distribution, to determine prevalence and potential infection hazards and to develop effective prophylactic measures. Studies on melioidosis are being conducted to evaluate its public health and military medical significance in SEASIA.					
9. KEY WORDS Immunology, serology, malaria, ecology, antibody, rickettsia, Thailand, Vietnam, wounds, bacteria, plague, leptospirosis, melioidosis, SEASIA.					
10. SUPPORTING PROJECTS Not Applicable					
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet			12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet		
DA FORM 1309R 1 June 63 PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of ____					

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

- (P) Yager, R. H., Col, VC, Div of Vet Med, WRAIR, WRAMC, Washington, D. C., 20012
576-3519 or Interdepartmental Code 198, Ext 3519 49
- (P) Hunter, D. H., Lt Col, MSC, Dept of Bacteriology, Div of Comm Dis and Immunology, WRAIR, WRAMC, Washington, D. C., 20012
576-3758 or Interdepartmental Code 198, Ext 3758 49
- (P) Elisberg, Bennett L., M.D., Dept of Rickettsial Diseases, Division of Communicable Disease and Immunology, WRAIR, WRAMC, Washington, D. C., 20012
576-2146 or Interdepartmental Code 198, Ext 2146 49
- (A) Diggs, C. L., Capt, MC, Dept of Medical Zoology, Division of Communicable Disease and Immunology, WRAIR, WRAMC, Washington, D. C., 20012
576-3361 or Interdepartmental Code 198, Ext 3361 49
- (A) Wellde, B. T., M.S., Department of Medical Zoology Division of Communicable Disease and Immunology, WRAIR, WRAMC, Washington, D. C., 20012
576-3361 or Interdepartmental Code 198, Ext 3361 49
- (A) Alexander, A. D., Ph.D., Dept of Veterinary Microbiology, Div of Vet Med, WRAIR, WRAMC, Washington, D. C., 20012
576-3518 or Interdepartmental Code 198, Ext 3518 49
- (A) Baker, H. J., Col, MC, Special Assistant to the Director, Walter Reed Army Institute of Research, WRAMC, Washington, D. C., 20012
576-3518 or Interdepartmental Code 198, Ext 3518 49
- (A) Keefe, T. J., Capt, VC, Dept of Vet Med, U. S. Army SEATO Medical Research Laboratory, Bangkok, Thailand 80
- (A) Ellison, D. W., 1/Lt, VC, USAMRU, c/o Institute of Medical Research, Kuala Lumpur, Malaya 79
- (A) Evans, L. B., B.S., Dept of Veterinary Microbiology, Div of Vet Med, WRAIR, WRAMC, Washington, D. C., 20012
576-3506 or Interdepartmental Code 198, Ext 3506 49
- (A) Dorsey, A. E., M.S., Dept of Veterinary Microbiology, Div of Vet Med, WRAIR, WRAMC, Washington, D. C., 20012
576-3506 or Interdepartmental Code 198, Ext 3506 49

ACCESSION NUMBER

36196

ARMY RESEARCH TASK REPORT

Continuation Sheet

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

- (A) Warner, A. R., Jr., Dept of Veterinary Pathology,
Div of Vet Med, WRAIR, WRAMC, Washington, D. C., 20012
576-3601 or Interdepartmental Code 198, Ext 3601 49
- (A) Baker, M., B.A., Dept of Veterinary Microbiology,
Div of Vet Med, WRAIR, WRAMC, Washington, D. C., 20012
576-3518 or Interdepartmental Code 198, Ext 3518 49
- (A) Cavanaugh, D. C., Major, MSC, Dept of Bacteriology,
Div of Comm Dis and Immunology, WRAIR, WRAMC,
Washington, D. C., 20012
576-3758 or Interdepartmental Code 198, Ext 3758 49
- (A) Rust, J. H., PhD, Dept of Bacteriology, Div of Comm
Dis and Immunology, WRAIR, WRAMC, Washington, D.C., 20012
576-3758 or Interdepartmental Code 198, Ext 3758 49
- (A) Sanders, T., SFC, Dept of Bacteriology, Div of Comm
Dis and Immunology, WRAIR, WRAMC, Washington, D.C., 20012
576-3758 or Interdepartmental Code 198, Ext 3758 49
- (A) Cary, S. G., M.S., Dept of Bacteriology, Div of Comm
Dis and Immunology, WRAIR, WRAMC, Washington, D.C., 20012
576-3758 or Interdepartmental Code 198, Ext 3758 49
- (A) Harkins, C., Dept of Bacteriology, Div of Comm Dis and
Immunology, WRAIR, WRAMC, Washington, D. C., 20012
576-3758 or Interdepartmental Code 198, Ext 3758 49
- (A) Fusillo, M. H., D. C. General Hospital, Washington, D. C.,
LI 7-9200 49
- (A) Noyes, H. E., PhD, Dept of Bacteriology, U. S. Army SEATO
Medical Research Laboratory, Bangkok, Thailand 80
- (A) Bozeman, F. Marilyn, M.S., Dept of Rickettsial Diseases,
Div of Comm Dis and Immunology, WRAIR, WRAMC,
Washington, D. C., 20012
576-3658 or Interdepartmental Code 198, Ext 3658 49
- (A) Rapmund, Garrison, Major, MC, Dept of Rickettsial Diseases,
Div of Comm Dis and Immunology, WRAIR, WRAMC,
Washington, D. C., 20012
576-2146 or Interdepartmental Code 198, Ext 2146 49

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

(A) Campbell, Janis M., U.S., Dept of Rickettsial Diseases,
Div of Comm Dis and Immunology, WRAIR, WRAMC,
Washington, D. C., 20012
576-3419 or Interdepartmental Code 198, Ext 3419 49

(A) Sangkasuvana, Vichai, Capt, MC, Royal Thai Army,
Thai Component, SEATO Medical Research Laboratory,
Bangkok, Thailand 80

(A) Scanlon, John E., Major, MSC, Dept of Entomology,
U. S. Army SEATO Medical Research Laboratory,
Bangkok, Thailand 80

REPORTS. Annual Progress Report, Walter Reed Army Institute
of Research, 1 July 1963 - 30 June 1964

Diggs, C. L. Comments on the Antigenic Analysis of Plasmodia.
Am. J. Trop. Med. & Hyg., 13:217-18, 1964.

Diggs, C. L., and Sadun, E. H. Cross-reactivity Studies in
Human Malaria by use of a Modified Fluorescent Antibody Test.
J. of Parasit. 49: (Suppl) 142, 1963.

Sadun, E. H. Introduction to International Panel Workshop on
Cultivation of Plasmodia and Immunology of Malaria.
Am. J. Trop. Med. & Hyg., 13:145-146, 1964.

Baker, H. J., Baker, M. F., Alexander, A. D., and Yager, R. H.:
Distribution of Pathogenic Leptospiras in Malayan jungles.
Bact. Proc. 1964, A.S.M.(RTI), p. 145.

Rubin, H. L., Alexander, A. D., Yager, R. H.: Melioidosis -
A Military Medical Problem. Mil. Med. 128: 538-542, 1963.

ANNUAL PROGRESS REPORT

Project No. 3A025601A811

Title: Military Medical Research
Program S. E. Asia

Task No. 01

Title: Mil Med Rsch Prog S. E. Asia

Subtask No. 21

Title: Mil med rsch prog, SEASIA (WRAIR)

Description: One object of this task is to seek needed information on the immunology, physiology, and pathogenesis of malaria toward the goal of gaining a better understanding of host specificity, natural and acquired resistance and host response to active and passive immunization. Additional objects include elucidation of epidemiologic, ecologic, serologic, bacteriologic, immunologic and other factors of infectious diseases of real or potential military importance in SEASIA.

Progress:

1. Pasteurella pestis antibody in human and rodent sera from Vietnam. Rodent sera collected over the past several years from plague endemic areas have been tested for complement fixing and hemagglutinating antibodies to the purified capsular antigen of the plague bacillus. These tests are specific for P. pestis antibody. The results obtained to date suggest that certain conclusions may be drawn from the results of these two serological determinations on sera from animals in a given area.

a. Animals which have been recently infected may have complement fixing antibodies.

b. Animals which have been infected some time in the past may have hemagglutinating antibodies but usually do not react in the complement fixation test.

c. Animals demonstrating both complement fixing and hemagglutinating antibodies are residents of active plague foci which have been operational for some time.

During the fall and spring quarters of 1962-63, it became evident that plague was present in Saigon. Several cases were confirmed by Major Feeley, 20th Medical Laboratory, and isolation of P. pestis was made from rat-borne fleas. At our suggestion a collection of rat sera from various areas of Saigon was made. These sera were all from Rattus norvegicus.

Results of complement fixation and hemagglutination tests performed on 409 R. norvegicus sera which were trapped in Saigon are given in Table I. These data indicate that at least two and possibly three distinct plague foci exist in Saigon and that rodent plague had occurred in much of that city. One case of plague was confirmed in Area I, two cases in Area II, and information indicated that a single case had occurred in Area III. Area III incorporated the Da Kao area of Saigon which has been an important problem area in previous years.

These serological studies were conducted to determine whether or not the extent of the epizootic could be determined by testing for P. pestis antibody. Since plague is a disease of rats, it can be expected that many rats will be exposed to infection in endemic or epizootic areas. Unfortunately, all areas were not trapped during the entire period and the number of sera secured from some of the areas was small, therefore, chronological conclusions pinpointing the course and extent of the epizootic cannot be made with a great deal of certainty, however, recent active plague foci can be delineated from the data presented in Table I. Areas I and II are areas that we believe had been operational for some time while Area III is an area that appears to have been recently invaded by the disease.

One hundred and ten pairs of sera collected from U. S. Army personnel recently returned from duty in South East Asia were tested for antibody to the Fraction 1 antigen of P. pestis and the polysaccharide antigen of Pasteurella tularensis. These personnel had been bled prior to departure and upon return from their mission. The complement fixation tests for tularemia antibody were entirely negative. Several (21 of 220) sera demonstrated complement fixing antibody to Fraction 1 and (81 of 220) sera had hemagglutinating antibody to the plague antigen. Several individuals demonstrated antibody titer rises. Since information as to the course and time of vaccination and bleeding dates is not available, the data cannot be interpreted.

2. Viability studies on enteric bacteria in a semi-solid transport medium. In a study initiated with the SEATO Medical Research Laboratory (U. S. Component), Bangkok, Thailand (Ann. Rpt., WRAIR, 1963) on the collection and shipment of fecal specimens in transport medium, the isolation of Salmonellae and Shigellae after 10-12 days was comparable. However, there was a considerable drop in the number of enteric pathogens isolated after storage for longer periods of time although Shigella flexneri 4 and Shigella boydii 4 were recovered from specimens after 59 days and 20 days storage, respectively. Vibrio comma Heiberg Groups III and IV were recovered for as long as 21 days. To better evaluate the results obtained in the preliminary trial with the SEATO laboratory, our laboratory, cooperating with D. C. General Hospital, Washington, D. C., compared the survival of shigella and salmonella in this medium. One hundred and sixty-two specimens (162) were

TABLE I
Plague Antibody in Sewer Rats (Rattus norvegicus)
Saigon, Vietnam - 1962-1963..

Area	Sample site	<u>Xenopsylla</u> <u>cheopis</u> index	Number rat sera tested	Number + reactors	
				CF	HT
I *	CB	0.29	26	3	1
	NCT	0.57	14	2	0
	COL	1.00	15	2	1
	CG	0.79	40	4	4
	BV	0.49	32	7	4
	BT	0.36	30	11	1
	CK	0.98	22	3	1
	NTC	0.95	9	2	0
II *	PTG	0.86	11	3	1
	LVD	0.74	8	2	0
	CVC	0.69	7	1	0
	M	****	3	0	2
	LTT	1.67	37	2	4
	NTT	1.20	35	8	3
III *	TQK	0.40	16	5	3
	YD	0.45	12	4	0
	TKC	0.38	16	5	0
	TMG	0.53	17	2	0
	NBK	0.98	35	8	0
IV **	BHT	0.03	9	0	0
	NCC	1.15	3	0	0
	THD	-	3	0	0
V ***	KTD	-	3	0	0
	CDT	-	2	0	1
	TQD	-	4	1	0
Totals			409	75(18.3%)	26(6.4%)

* Highly suspicious areas
 ** Negative area
 *** Geographic locale not certain
 **** Not given

collected. The shigella strains isolated were S. sonnei I and II and S. flexneri 5. Salmonella isolated included S. typhi-murium, S. saint-paul, S. enteritidis and S. manhattan. There was 100 per cent correlation between our findings and those of the hospital laboratory. After storage of specimens in transport medium at room temperature (approximately 20°C) the shigella and salmonella were recovered for 45 days even though there were large members of Proteus sp. and Pseudomonas aeruginosa present. In other specimens not containing large numbers of these organisms, salmonella were recovered for periods up to 62 days.

Several swabs from both cultures of a ferrous sulfate dependent P. pestis strain were placed in this new holding medium and maintained at room temperature. The plague bacillus has remained viable and recoverable in large numbers for over seven months. If field trials using clinical material support these preliminary observations, this transport medium would prove to be a valuable adjunct to field technics for the study of plague foci.

During the past year, several hundred vials of transport medium were shipped to Lt. Col. Oscar Felsenfeld in India and to a University of Maryland medical group in Pakistan for the collection and shipment of fecal specimens during a cholera epidemic. Lt. Col. Sidney Gaines has also shipped supplies of this transport medium to a group in South America for further field use.

3. Bacteriologic survey of traumatic wounds in South Vietnam.
In support of a team organized for conducting a survey among Vietnam battle casualties, a study was made to determine the qualitative and quantitative bacterial flora of wounded soldiers and to relate findings to such parameters as weaponry, prior therapy, evacuation, etc. Other portions of the study consisted of obtaining serum samples for immunologic assays and determining the antibiotic sensitivity of selected organisms.

Most specimens were from patients at Tong Y Vien Cong Hoa Military Hospital at Saigon. The remainder were from a province hospital at Cantho, approximately 80 miles southwest from Saigon. Evacuation of wounded was usually by helicopter or jeep ambulance. If the former, patients were transported from the helicopter pad to the triage room by ambulance. Most initial cultures were taken in the triage room, while subsequent cultures were taken on wards.

Wound cultures were taken by inserting sterile standard machine-rolled swabs deep into wounds until the cotton was saturated. Each cotton tip was broken off into a 9.0 ml screw capped vial containing 0.9 ml of 2 per cent trypticase. These were immediately taken to the laboratory for bacteriologic processing. The following media were

employed with each of the specimens:

MacConkey agar for gram negative bacilli.

S. F. medium containing 2 per cent agar for fecal streptococci.

Mannitol Salt Agar for staphylococci.

Blood agar (5 per cent human blood) for total aerobic flora.

Blood azide egg yolk agar incubated anaerobically for counting histotoxic clostridia.

Counts of bacteria in all cultures were carried out to the nearest log by the streak dilution technique. At the conclusion of the bacteriological studies all data were encoded on punch cards for analysis.

Antibiotic sensitivity studies were carried out by noting zones of inhibition around pure culture isolates of selected organisms. Sera for immunologic studies were obtained from volunteer donors at the Vietnam Army Blood Bank and from mountain tribesmen from the province of Phu Bon (these sera made available by Major Eugene Feeley, MC). Assays for clostridial antitoxins were made by testing for blockade of the Lecitho-vitellin (L.V.) test or by use of the hemolysis indicator plate of Easterling.

Therapy of patients was the responsibility of the Vietnam Army Medical staff and varied according to available supplies and the responsible physician. Patients with wounds likely to develop tetanus were usually injected intramuscularly with 1500 units of tetanus antitoxin as a prophylactic measure even though all members of the military have been immunized against this disease.

Findings were based on a total of 202 specimens from 131 individuals. The bacteriologic studies were arbitrarily designed to preclude organisms from consideration as infecting agents unless they were present in counts of $\log 10^3/\text{ml}$. It is realized that such a technique has the limitation of disregarding potential pathogens when fewer than $\log 10^3/\text{ml}$ of wound exudate but attempts were made to compensate for this limitation by obtaining subsequent samples on patients developing overt infections.

Qualitative results in Table II indicate that the most efficient colonizers of wounds were staphylococci as evidenced by the high percentage of early wounds containing 1,000 or more organisms/ml of exudate. By contrast it seems certain that most infections with Pseudomonas sp were acquired in the hospitals. The relative resistance

TABLE II

Bacteria Most Frequently Isolated From Wounds
(Expressed to nearest percent of total positive cultures)

Sample Time (Hours after Wounding)	No. of Positive Cultures	Organism						
		Staphylococcus	Streptococcus fecalis	Coliforms	Bacillus	Pseudomonas	Clostridia	Others
8	35	83	34	37	26	3	6	3
9-24	29	76	79	41	14	10	21	0
24	30	73	30	27	23	27	16	3
During Hospitalization	37	58	38	51	19	35	16	3

of this organism to available antibiotics (Polymyxin B was unavailable) precluded specific therapy for established pseudomonas infections except for the use of autogenous vaccines of dubious value. Clostridia were present in 16 wounds in counts of 100,000/ml of exudate - certainly enough to be considered an established infection. These high counts persisted for days with no evidence of clinical gas gangrene.

Quantitative bacteriological findings were related to a number of parameters. The elapsed time from wounding to obtaining the initial cultures (Table III) reaffirm the value of early evacuation of wounded because there was an inverse ratio between the elapsed time and the bacterial counts of wounds.

Analysis of bacterial counts of different types of wounds (Table IV) indicated that penetrating wounds were most likely and perforating wounds least likely to be infected. Results in Table V show that most infections resulted when the wounding agent was a grenade and little difference was noted in bacterial counts with the other weapons listed.

Only three small puncture type spike wounds were seen. No bacteria were cultured from one wound and the only organisms found in the other two were aerobic gram positive cocci. All three were treated with tetanus antitoxin and antibiotics and were discharged within five days of admission. Two iron spike devices were cultured for anaerobes, and toxigenic clostridia were readily isolated from both. The spikes on these devices were rusty and it would have been surprising if clostridia had not been present.

The data in Table VI relating bacterial counts to the anatomical site of the wound show that infections of the areas distal to the elbows and knees were most likely to be infected. More than half of the wounds of the lower legs and feet were from mine explosions which presumably carried an inoculum of dirt into the wound. Analyses of other parameters such as weaponry, types of wounds and evacuation times provide no ready explanation for the decreased bacterial counts of wounds of the upper arms, shoulders, upper legs and thighs.

The data in Table VII indicate that there was no appreciable effect of antibiotic therapy on bacterial flora. These data are misleading in that resampling was not done on a random basis; rather the choice was based on wounds that (1) on initial sampling seemed likely to develop infection and (2) showed evidence of infection at the time of resampling. Perhaps the decrease from 124 to 24 samples is indirectly a testimonial to the adequacy of antimicrobial therapy.

Antibiotic sensitivities of selected wound isolates are shown in Table VIII. All sensitivities were based on the disks with an antibiotic concentration compatible with attainable blood levels. These values

TABLE III

Bacterial Counts of Wounds Relative to Time of Wounding

Sample Time Hrs. after Wounding	No. of Specimens	Log # Bacteria Expressed as percent of total specimens counted								
		<3	3	4	5	6	6.5	7.0	7.5	>7.5
0-4	35	66	11	11	3	9	0	0	0	0
5-8	42	52	5	14	9	9	5	0	0	0
9-24	43	44	5	14	14	9	5	9	0	0
>24	39	28	10	10	13	26	3	8	0	3
Resamples	43	19	5	5	9	19	19	5	16	2

TABLE IV

Bacterial Counts of Wounds Relative of Type of Wound

Type of Wound	No. of Specimens *	Log # Bacteria Expressed as percent of total specimens counted								
		<3	3	4	5	6	6.5	7.0	7.5	>7.5
Penetrating	90	40	8	17	12	12	2	9	0	0
Perforating	54	69	6	6	7	7	2	2	0	2
Mutilating	18	50	0	11	11	11	11	6	0	0

*Initial specimens only

TABLE V

Bacterial Counts of Wounds Relative to Wounding Agent

Wounding Agent	No. of Specimens*	Log # Bacteria Expressed as percent of total specimens counted								
		<3	3	4	5	6	6.5	7.0	7.5	>7.5
Rifle or Machine Gun	67	51	4	13	6	16	1	6	0	1
Grenade	27	37	4	19	22	11	0	7	0	0
Mine	59	54	8	10	10	5	5	7	0	0
Other (Spikes Secondary Missiles)	12	50	8	8	0	17	8	8	0	0

*Initial specimens only

TABLE VI

Bacterial Counts of Wounds Relative to Location of Wound

Location of Wound	No. of Specimens	Log # Bacteria Expressed as percent of total specimens counted								
		<3	3	4	5	6.0	6.5	7.0	7.5	>7.5
Head, neck, throat and abdomen	16	44	0	25	6	12	0	12	0	0
Buttocks	12	42	0	17	17	17	0	8	0	0
Upper arms and shoulders	13	77	0	8	8	0	0	8	0	0
Lower arm and hand	26	46	8	15	15	8	4	4	0	0
Upper leg and thigh	43	63	14	5	5	5	0	2	5	2
Lower leg and foot	55	38	4	11	18	15	5	7	2	0

TABLE VII

Bacterial Counts of Wounds Relative to Prior Antimicrobial Therapy

Prior Rx	No. of Specimens	Log # Bacteria Expressed as percent of total sample counted								
		<3	3	4	5	6.0	6.5	7.0	7.5	>7.5
No	124	62	6	15	10	8	2	7	0	0
Yes	24	54	13	0	17	4	4	4	0	4

TABLE VIII

Relative Sensitivity of Wound Isolates to Various Antibiotics

Organism	No. of Cultures	Percent of Cultures Sensitive to -							
		Penicillin (2 units)	Erythro- mycin (2 γ)	Caloram- phenicol (5 γ)	Novo- biocin (5 γ)	Tetra- cycline (5 γ)	Kana- mycin (5 γ)	Neo- mycin (5 γ)	Strepto- mycin (2 γ)
<u>Staphylococcus</u> sp. (Initial Specimens)	72	47	86	47	69	24	7	1	3
<u>Staphylococcus</u> sp. (Resamples)	17	6	71	47	100	18	6	12	0
<u>Clostridium</u> sp.	22	64	68	95	32	95	0	0	0
<u>Pseudomonas</u> sp.	8	0	0	25	0	0	13	0	0
<u>Streptococcus</u> <u>fecalis</u>	10	0	0	0	0	20	40	0	0
Coliforms	10	0	0	60	0	0	40	60	20

represent inhibitory rather than bactericidal levels. The percentage of staphylococci resistant to penicillin was not surprising in view of the general usage of that drug. However, the relative ineffectiveness of tetracycline was a surprise. Chloramphenicol, which was the most used broad spectrum antibiotic at Cong Hoa, seems to represent a good choice. Erythromycin and novobiocin would appear to be good agents to be held in reserve for staphylococcal infections refractory to penicillin and the tetracyclines. A modest stockpile of the newer biosynthetic penicillins might be indicated should erythromycin and novobiocin become readily available in that area.

Qualitative analysis of sera of presumably normal soldiers and civilians in Vietnam showed that 44 soldiers had circulating antitoxin against the alpha toxin of C. perfringens and 32 against the gamma toxin of C. novyi (Table IX). The importance of this finding is unknown because no comparable studies are available for comparison. None of the individuals had a history of gas gangrene and all indicated that they had never received clostridial antitoxin. All, however, had been immunized against tetanus with an apparently effective toxoid, and studies carried out some years ago in this laboratory suggest that there was some interrelationship between tetanus immunization and susceptibility to gas gangrene. A more logical explanation is that the individuals with demonstrable antitoxin had some prior experience with low grade clostridial infection. Credence is given to this possibility by the finding that a number of patients had most of the ingredients for classical gas gangrene, i.e., inoculum, trauma, devitalized tissue and foreign bodies, but only one developed severe clostridial myonecrosis. In that instance the circulation was severely impaired by a tight tourniquet consisting of a portion of automobile inner tube. A second patient with necrotic tissue and crepitation responded favorably to antibiotic therapy in the absence of surgical debridement. An explanation for the absence of antitoxin to the alpha toxin of C. septicum is that this organism was never isolated from any wound (Table X).

Most patients with wounds involving muscle received tetanus antitoxin and some received intramuscular gas gangrene antitoxin of unknown composition. Thus possible explanations advanced for the paucity of cases of gas gangrene in this area are (1) the presence of circulating gas gangrene antitoxins resulting from administration, prior low grade infections or cross protectiveness of tetanus antibodies, (2) relatively avirulent toxigenic clostridia and (3) rapid evacuation of patients. Lack of inoculum, absence of trauma, devitalized tissues and foreign bodies are not possible explanations.

TABLE IX

Antibody Spectrum of Human Sera from Vietnam Nationals
(Expressed to nearest percent of total sera assayed)

Antibody Identified	Technique Used	Source of Sera	
		Vietnam Soldiers (103)	Mountain Tribesmen (22)
<u>Clostridium</u> <u>perfringens</u> Alpha toxin	Hemolysin Inhibition and Lecithinase Inhibition	44	0
<u>Clostridium</u> <u>novyi</u> Gamma toxin	Hemolysin Inhibition	32	0
<u>Clostridium</u> <u>septicum</u> Alpha toxin	Hemolysin Inhibition	0	0

TABLE X

Clostridial Species Isolated from War Wounds in South Vietnam

Clostridium species	Percentage of Wounds from which this Organism was Isolated
<u>C. perfringens</u>	13
<u>C. sporogenes</u>	9
<u>C. novyi</u>	6
<u>C. sphenoides</u>	6
<u>C. sordelli</u>	4
<u>C. histolyticum</u>	2
<u>C. fallax</u>	2
<u>C. septicum</u>	0

4. Serological cross reactivity between *P. vivax* and *P. falciparum* as determined by a modified fluorescent antibody test. A study was undertaken to determine to what extent a cross reactivity between *Plasmodium vivax* and *P. falciparum* can be detected in the fluorescent antibody (FA) test. The relative specificity of the FA test employing these two parasites as antigens was also compared.

The specimens examined were of three types: 1) dried blood from proven natural human infections on filter paper, 2) sera from experimentally infected human volunteers, and 3) sera from individuals with naturally acquired malaria.

The diagnosis of malaria in various patients was established by the recovery and identification of organisms from the blood. To determine the relative specificity of the FA test employing *P. vivax* and *P. falciparum* as antigens, control sera with bacterial, mycotic and parasitic infections other than malaria were used. To test whether the presence of autoantibodies would give rise to false positive reactions, specimens from patients with lupus erythematosus and with rheumatoid arthritis were included. Normal sera were obtained from healthy American donors.

Eighty specimens of blood from natural infections on filter paper were tested against *P. vivax* and *P. falciparum* as sources of antigen. Two separate dried blood samples were pooled after extraction to insure that equal blood concentrations were reacted against each antigen. Of these, 50 gave positive reactions. Of the 29 positive *P. vivax* sera, 6 reacted with *P. falciparum* also. Among the 21 positive *P. falciparum* sera 9 reacted with both *P. vivax* and *P. falciparum* antigens while two reacted with *P. vivax* only. The results are summarized in Table XI.

Table XI
Cross Reactivity in Filter Paper Blood Specimens
from Natural Infections

Type Reaction	Infection	
	<i>P. vivax</i>	<i>P. falciparum</i>
Homologous only	23	10
Homologous and Heterologous	6	9
Heterologous only	0	2
Total	29	21

Since samples obtained from endemic areas do not permit to ruling out the possibility that some of the donors might have been previously infected by the heterologous parasite, no firm conclusion could be made on the degree of cross reactivity of the two species. Therefore, further studies were performed on specimens from volunteers at the Army Medical Research Project of the University of Chicago who had not previously been exposed to malaria. Twelve sera from volunteers with P. falciparum infections and nine sera from P. vivax infections were titrated. The results summarized in Table XII supported the previous findings and indicated that although sera reacted at higher titers when the homologous antigen was employed, extensive cross reactions occurred.

Table XII
Frequency Distribution of FA Titers (First series)

Antigen	Antiserum	Reciprocal of Titer								GMT*
		0	5	10	20	40	80	160	320	
<u>P. falciparum</u>	<u>P. falciparum</u>	0	2	1	3	3	2	0	1	28.3
	<u>P. vivax</u>	3	2	1	1	0	2	0	0	9.3
<u>P. vivax</u>	<u>P. vivax</u>	0	1	3	2	3	0	0	0	17.2
	<u>P. falciparum</u>	3	4	3	2	0	0	0	0	6.3

*Geometric mean titer

A second series of nine P. falciparum and six P. vivax sera from human volunteers was examined similarly at a later date. The results (Table XIII) confirmed the presence of broad cross reactivity. Eighteen titrations of P. falciparum sera and twelve P. vivax sera are shown. The titrations on one-half of each of these groups was performed on plasma while the other half was performed on matched blood samples collected on filter paper at the time the plasma was collected to permit a comparison of the two collection methods. As shown in the table, there is less than a two-fold dilution difference in titrations on the specimens collected by the two methods. The large number of negatives can be explained in part by the high initial dilution (1:20) which was necessary due to limited amounts of filter paper eluate.

Table XIII
Frequency Distribution of FA Titers (Second series)

Antigen	Antiserum	Reciprocal of Titer											GMT*
		0	20	40	80	160	320	640	1280	2650	2650	GMT*	
<u>P. falciparum</u>	P.	3	0	4	2	2	1	3	2	1	1	13.2	
	<u>falciparum</u>												
	<u>P. vivax</u>	9	3	0	0	0	0	0	0	0	0	11.9	
<u>P. vivax</u>	<u>P. vivax</u>	2	5	2	2	1	0	0	0	0	0	30.0	
	<u>P. falciparum</u>	10	3	1	3	1	0	0	0	0	0	20.0	
Both	Both	8	7	5	4	2	0	1	2	1	1	41.0	
	(Plasma)												
Both	Both	16	4	2	3	2	1	2	0	0	0	24.2	
	(Filter paper)												

*Geometric mean titer

A fourth experiment was designed to compare the relative specificity of the FA test when P. vivax and P. falciparum were used as antigens. A total of 152 sera were divided into two aliquots and tested with each of these two antigens. The results (Table XIV) suggest that a greater degree of specificity was possible when P. vivax was used as antigen. This was not necessarily due to a corresponding lower sensitivity of this antigen, since the number of positive reactions obtained with these two antigens in endemic areas was not significantly different.

In general, the data presented indicate that the fluorescein-labeled antibody technic using either P. vivax or P. falciparum as antigen can be successfully employed in the laboratory diagnosis of human malaria. The relatively few positive reactions obtained with sera from individuals with bacterial, mycotic and parasitic infections and with collagen diseases suggest that a high degree of generic specificity is possible in the test. Attention is called to the fact that since many of the serum specimens used in the specificity studies were obtained in areas where malaria has been known to occur, the few positive reactions observed with sera from malaria-free patients may not necessarily indicate "false" positives. However, it is of interest that a small percentage of malarial sera have been found reactive in FA tests using trypanosomes as antigen. Fewer cross reactions were observed with P. vivax antigen than with P. falciparum. However, the relative reliability of this test with these two antigens must await more complete documentation.

As observed for schistosomiasis, trypanosomiasis and leishmaniasis, these results indicate that minute amounts of dried blood specimens obtained in endemic areas on absorbent paper can be tested satisfactorily for malaria antibodies by the FA technic after they have been transported to a distant laboratory.

5. The physiological pathology of Plasmodium berghei infections in mice. The scarcity of information on the physiological changes produced by various species of malarial parasites is probably due to the fact that biochemical studies in naturally infected humans in endemic areas give results which are difficult to interpret in view of concomitant infections and nutritional deficiencies. Well controlled studies in experimental animals have been seriously handicapped until recently by the lack of available technics which could employ minute amounts of blood and still retain a high degree of accuracy. Studies conducted with recently developed micro-technics have indicated that a great reliance can be placed on their accuracy and that extremely minute amounts of blood are needed for these tests, thus

permitting the determination of some of the effects produced by Plasmodium berghei in mice and rats.

In a preliminary series of experiments the following determinations were conducted in mice infected with P. berghei and in uninfected controls: chlorides, calcium, creatinine, glucose, nonprotein nitrogen, alkaline phosphatase, acid phosphatase, SGOT transaminase, phosphorus, carbon dioxide, total proteins and serum electrophoresis. The results of these preliminary experiments are summarized in Table XV. In general, they indicate that infected animals had significantly lower values of chloride, glucose, alkaline phosphatase, phosphorus and total protein. Significant increases in infected mice were observed in the SGOT transaminase and carbon dioxide. Studies on the relative proportions of serum proteins (Table XVI) indicated that the lower values of total proteins in the infected animals could be accounted for by a markedly decreased amount of albumin. No significant differences were found in the amounts of alpha 1, alpha 2, beta and gamma globulins. These studies are being continued and extended to include investigations with human volunteers.

6. Determination of Plasmodium berghei density for inoculum preparation. In order to improve the standardization of inocula for use in experimental Plasmodium berghei infection, a method for the direct enumeration of the parasites has been devised.

A 1:100 dilution of heavily parasitized blood was made with a solution of 0.05% Nile blue sulfate (C.I. 51180) in a saline citrate solution (sodium chloride 7.4 gms/l, trisodium citrate dihydrate 9.1 gms/l, and citric acid 0.6 gms/l) in a red blood cell pipette. The contents were agitated, allowed to stand for fifteen minutes, agitated again and a hemocytometer chamber was filled. The number of parasitized erythrocytes in one square millimeter of the 0.1 millimeter thick chamber is then determined and the concentration of parasites in the original blood sample computed. The latter is then diluted to provide an inoculum of the desired parasite concentration.

This method avoids the necessity for determination of per cent parasitemia from a thin smear and of a second determination of the red blood cell count with subsequent calculation of the parasite concentration. However, with the present procedure, morphological differentiation of the elements of the blood is more difficult than with Romanovsky stains. Replicate counts on single specimens indicate that the technic is more reproducible than the determination of per cent parasitemia from conventional thin blood smears.

Table XIV
Specificity Studies Using P. vivax and P. falciparum as Source
of Antigen

Diagnostic Status	Number Tested	Number Positive	
		<u>P. vivax</u> Antigen	<u>P. falciparum</u> Antigen
Presumptive Malaria (Nigeria)	29	26	23
Other protozoan diseases	35	8	14
Leishmaniasis donovani	10	4	6
Leishmaniasis braziliensis	11	0	1
Leishmaniasis tropica	1	0	0
Trypanosomiasis rhodesiensis	10	4	7
Trypanosomiasis cruzi	2	0	0
Amebiasis	1	0	0
Other communicable diseases	57	1	5
Syphilis	13	0	0
Tuberculosis	10	0	0
Histoplasmosis	8	0	0
Coccidioidmycosis	2	0	0
Strongyloidiasis	6	0	0
Schistosomiasis mansoni	3	0	0
Schistosomiasis haematobia	8	0	0
Onchocerciasis	7	1	5
Collagen diseases	11	0	0
Lupus erythematosus	9	0	0
Rheumatoid arthritis	2	0	0
Healthy US Males	20	0	0
Totals excluding Nigerian sera	123	9	19

Table XV

Results of Clinical Pathological Determinations in Mice Infected with Plasmodium berghei

Infection Group	No. Mice	Mean Values of Biochemical Tests							
		Chlor. (mg%)	Ca (mg%)	Creatin. (mg%)	NPN (mg%)	Glucose (mg%)	Alk. Ph. (units)	Acid Ph. (units)	SGOT Phosph. (units) (mm/l)
<u>P. berghei</u>	7	100.2	9.7	1.0	39.4	40.3	2.9	2.6	122 7.6 26.0
Healthy Controls	7	114.7	9.4	0.9	43.5	124.3	6.7	2.6	67 11.1 18.9

8888

Table XVI

Amounts of Serum Proteins in Mice Infected with Plasmodium berghei

Infection Group	No. Mice	Results of electrophoretic determinations in gm %					
		Total Protein (mg %)	Albumin	α_1 globulin	α_2 globulin	β globulin	globulin
<u>P. berghei</u>	7	4.7	1.9	0.6	0.6	1.5	0.1
Healthy controls	7	5.4	3.1	0.6	0.5	1.1	0.1

7. Immunodiffusion analysis of Plasmodium berghei. In order to gain some insight into the mechanisms of immunity in malaria, immunodiffusion analysis of Plasmodium berghei has been performed. Parasites were obtained from mice which had been inoculated with 10^7 parasitized erythrocytes from mice infected a week earlier.

The soluble extract of the parasites was prepared by addition of two volumes of cold 0.05 M sodium diethyl barbituate-hydrochloric acid buffer, pH 8.2 to the packed parasites and treatment of the mixture with 20 kc sound for four 30 second intervals.

Six rabbits were hyperimmunized with fresh extract in complete Freund's adjuvant by combined foot pad, intraperitoneal, and subscapular routes. These injections were given on three occasions approximately one month apart and the animals were bled one week later. An additional three rabbits were immunized using the whole homogenate as antigen by a similar schedule.

The nine sera prepared in this manner were analyzed by the agar gel diffusion technic (Ouchterlony) using the reconstituted extract as principal antigen. To detect the presence of antibodies directed against mouse antigens additional materials were employed. Mouse spleen was minced and extracted in the alkaline buffer by the use of ultrasound or a high speed homogenizer. This material was then subjected to high speed centrifugation and the supernatant used as antigen. Second and third mouse extracts were prepared by lysis of washed erythrocytes in ten volumes of distilled water with subsequent high speed centrifugation and collection of both the supernatant of liquid hemolysate and the pellet of stroma. The stroma was extracted by the same procedure outlined above for the spleen preparation. Another mouse antigen preparation was normal mouse serum.

The Ouchterlony analyses revealed a complex pattern with bands between each of the antigen preparations and one or more of the sera. No consistent differences were noted between the two groups of sera although individual rabbits varied independently. Preimmunization sera gave no reaction.

The most intense band was shared by all the mouse components as well as the plasmodial extract. It occurred early (3-4 hours) in the course of diffusion and antigen excess was observed within 24 hours, especially with the liquid hemolysate preparation. Immunelectrophoresis

in alkaline buffer revealed a diphasic mobility. A positive reaction of this component with benzidine identified the antigenic component as mouse hemoglobin.

A second component occurred only with a small number of antisera and normal mouse serum. This relatively weak component was studied by electrophoresis of normal mouse serum with subsequent simultaneous development of precipitin bands with the experimental antiserum and with antimouse serum. A comparison of the patterns revealed the band to be formed by mouse gamma-2 globulin.

The extracts of spleen and erythrocyte stroma also reacted with the antisera. A minimum of two bands confined to the spleen preparation and one band confined to the stroma were observed. These components, like the gamma-2 globulin band, were never observed in the reaction between the P. berghei preparation and the antisera. A minimum of two bands associated with the parasite were also observed. Additional Ouchterlony experiments using sera absorbed with an excess of hemolysate revealed obliteration of the hemoglobin line with resulting increased ease in interpretation of the remainder of the patterns. The multiplicity and distribution of the components was confirmed by these experiments.

The hemagglutinin activity of the antisera was examined. One of the sera was found to have a titer of 1/64 and was used for further study. When the antiserum was absorbed with an excess of hemoglobin so that it no longer gave a precipitin band with this antigen, no effect on its hemagglutinin activity was observed (Table XVII). Conversely, when the hemagglutinin titer was abolished by absorption with intact erythrocytes both the hemoglobin and stromal bands observed in Ouchterlony tests was abolished. Thus the hemagglutininogen is not hemoglobin but might be the antigen responsible for the band observed with erythrocyte stromal extract. Preimmunization sera gave no reaction. No further characterization was attempted.

Since the Ouchterlony tests described above did not allow comparison of the nine sera with respect to identity of specific P. berghei antigens, experiments were designed with a format allowing such comparison. For these studies the antigen was concentrated by 2 1/2 times. These experiments revealed a minimum of five different precipitin bands attributable to the parasitic antigens.

Table XVII
Effect of Hemoglobin Absorption on Hemagglutinin Activity of
Anti P. berghei Serum

Serum dilution	1/2	1/4	1/8	1/16	1/32	1/64	1/128
Unabsorbed serum	4	4	4	4	3	0	0
	4	4	4	4	3	0	0
	4	4	4	4	3	0	0
Absorbed serum	4	4	4	4	3	±	0
	4	4	4	4	±	0	0
	4	4	4	4	4	0	0

Sera were selected on the basis of the Ouchterlony analyses for use as reagents for the further study of the P. berghei components by immunoelectrophoresis at pH 8.2 at an ionic strength of 0.05.

These studies again revealed five precipitin bands when the concentrated antigen was reacted with varying amounts of the selected antisera. All of the components had a positive mobility as determined by parallel runs with dextran to determine the inherent electroendosmotic effect of the medium used (Ion #2). None of these components had a mobility greater than that of bovine serum albumin.

The bands were subjected to qualitative chemical tests for their further characterization. Two of the components give positive periodic acid Schiff reactions suggesting lipid or carbohydrate components. Basic fuchsin staining for carbohydrate gave negative results while oil red O tests for lipid were positive for both of the components.

Table XVIII summarizes the characteristics of the constituents of the P. berghei extracts in terms of the precipitating antibodies elicited.

These studies demonstrate that a soluble extract of P. berghei contains a number of materials of host origin, hemoglobin being quantitatively

Table XVIII

Constituents of Soluble P. berghei Extract as Studied by
Immunodiffusion with Rabbit Antisera

<u>Source of high concentration of constituent</u>	<u>Characterization</u>
Erythrocyte soluble phase (1 component)	<ol style="list-style-type: none"> 1. Associated with all mouse origin preparations tested. 2. Most rapid reaction with erythrocyte 3. Early antigen excess phenomenon with erythrocyte soluble phase 4. Disappears on abolition of hemagglutinin titer by absorption with intact erythrocytes 5. Migrates as two components with a fusion reaction in immunoelectrophoresis 6. Benzidine positive
Erythrocyte stromal extract (1 component)	<ol style="list-style-type: none"> 1. Associated only with stromal extract 2. Disappears on abolition of hemagglutinin titer by absorption with intact erythrocytes
Serum (1 component)	<ol style="list-style-type: none"> 1. Associated with serum only 2. Migrates as a gamma-2 globulin in immunoelectrophoresis (identical with band developed with rabbit anti-mouse serum)
Spleen extract (2 components)	<ol style="list-style-type: none"> 1. Associated with spleen extracts only
Mouse hemagglutininogen	<ol style="list-style-type: none"> 1. Activity not affected by hemoglobin absorption 2. Absorption of hemagglutinin also ablates hemoglobin and stromal bands
<u>P. berghei</u> soluble antigen (5 components)	<ol style="list-style-type: none"> 1. Associated with <u>P. berghei</u> preparations only 2. Mobilities positive at pH 8.2 3. Mobilities less than that of bovine serum albumin 4. Two components oil Red O positive (lipoproteins)

the most important of these. The characterization of specific parasite antigens provides a basis for the study of the molecular mechanisms of immunity to rodent malaria.

8. The relationship of size of inoculum to infections with *P. berghei* in mice. Because there is a lack of knowledge regarding basic host parasite relationships in *P. berghei* infections, studies were undertaken to define the dose response of the parasite in mice. The mice were inoculated intraperitoneally with single doses of *P. berghei* numbering from 1×10^7 to 1×10^8 parasitized cells. Counts were made by the Nile Blue Hemocytometer method. Ten-fold dilution from 1×10^1 were then carried out. None of the animals receiving a dose of 10^4 or less developed a detectable parasitemia. All animals receiving a dose of 10^6 or greater developed a parasitemia and subsequently died. At a dosage of 1×10^5 , male mice showed a 70% mortality while female mice had a mortality rate of 80%. The LD 50 of the male mice under these conditions was approximately 7×10^4 and that of the female mice was 6×10^4 (Table XIX).

Table XIX

The Dose Response of Male and Female Mice Inoculated with Varying Numbers of *Plasmodium berghei*

Size of Inoc.	Sex	Animals in Group		% Mort.	Range(Days)	MST*
		Alive	Dead			
10^8	male	0	10	100	5-7	6.0
	female	0	10	100	5-8	6.2
10^7	male	0	10	100	6-10	7.6
	female	0	10	100	7-8	7.5
10^6	male	0	10	100	7-9	7.9
	female	0	10	100	7-8	8.5
10^5	male	3	7	70	12-15	13.5
	female	2	8	80	12-22	17.0
10^4	male	10	0	0	-	-
	female	10	0	0	-	-
10^3	male	10	0	0	-	-
	female	10	0	0	-	-
10^2	male	10	0	0	-	-
	female	10	0	0	-	-
10^1	male	10	0	0	-	-
	female	10	0	0	-	-

*Mean survival time in days from inoculation to death

This study will be continued to more clearly define the host parasite relationship in regards to weight, age, and route of infection.

9. Comparisons of the course of infection with *P. berghei* in different animals. A search for a large host for *P. berghei* had been indicated by the large amounts of parasitic material needed for biochemical analyses and serological technics. Some larger animals have been tested as to their susceptibility to *P. berghei* as follows:

Rabbit	Insusceptible
Guinea pig (intact)	Insusceptible
Guinea pig (splenectomized)	Insusceptible
Gerbil	Susceptible
Muskrat	Very susceptible
Rhesus Monkey (splenectomized)	Moderately susceptible

The South African gerbil exhibits a susceptibility to *P. berghei*; self cure occurred with low dosages while large doses resulted in death. The muskrat was very susceptible with a parasitemia reaching 90%. Results in Rhesus monkeys were surprising as splenectomized animals were moderately susceptible. Parasitemia appeared approximately one week after inoculation, reached a peak of approximately 25,000 parasites per cubic mm and was demonstrable for about two weeks. Blood transfer of the parasite to other splenectomized Rhesus monkeys and to mice has been accomplished. This study will continue along with an investigation of various South American rodents (Capybara, Paca, Agouti, Nutria) and other large North American rodents (Woodchuck, Prairie dog).

10. Attempts to induce immunity to Plasmodium berghei following injection of parasites attenuated by ionizing radiation. Development of an acquired immunity in animals after inoculations of irradiated larvae of hookworm, cercariae of schistosomes and oocysts of coccidia has been demonstrated. Attempts are being made to determine if irradiated *P. berghei* parasites, when injected into rodents are capable of inducing a detectable acquired immunity. Mouse blood infected with *P. berghei* was diluted to a concentration of 1×10^7 parasitized cells per 0.1 ml of suspension. Aliquots of this suspension was made and irradiated at dosages of 5, 10, 15, 20, 30, 40 and 50 Kr in a gamma cell 220 cobalt 60 irradiator. Mice receiving parasitized cells irradiated at 5 Kr and 10 Kr developed a detectable parasitemia and death resulted. Roentgen dosages of 15 Kr and over produced no detectable parasitemia and no deaths in these groups occurred. Levels of irradiation between 10 Kr

and 15 Kr show that a dose of 13 Kr is sufficient to prevent death of the mice. Immunizations of 10^7 parasitized cells at 13 Kr were given intraperitoneally to mice. Groups immunized with 1 and 2 doses of irradiated parasites showed no parasitemia. However, those receiving over two immunizing doses developed a high parasitemia and death resulted. The mice which received 1 and 2 immunizing doses were challenged with 1×10^7 P. berghei with the following results:

<u>Immunizing Doses</u>	<u>Range (Days)</u>	<u>Mean Survival Time</u>
1. 1	6-12	9.1
2. 2	7-17	8.4
3. Controls	6-15	9.7

No protection was evident at this time. The study will continue using modifications of roentgen dosage, routes of immunizing doses and sizes of the challenging inoculum.

11. Attempts to induce immunity to Plasmodium berghei in mice following injection of homogenates of parasites. In order to explore the mechanisms of immunity to malaria, attempts were made to artificially induce resistance to Plasmodium berghei infections in white mice after injection of a parasite homogenate.

The homogenate was prepared by ultrasonic treatment of parasites separated from the host cell by saponin treatment as described previously. A control preparation consisted of an homogenate of packed normal erythrocytes treated in the same manner as the packed parasites.

In each of two experiments, three groups of male and three groups of female BALBC mice were selected randomly for treatment. Group I received 0.1 ml of an emulsion of the P. berghei homogenate in complete Freund's adjuvant. Group II received 0.1 ml of the control blood homogenate in complete Freund's adjuvant while Group III received 0.1 ml of the alkaline buffer used for preparing the homogenates. All injections were given intramuscularly. The treatments were given on day 0 and day 27 of the study. On day 31 the animals were challenged with 10^7 parasitized erythrocytes. Per cent parasitemias and the mean survival time of the animals were determined. These data are shown in Table XX.

Table XX
Effect of Homogenate on P. berghei Infections in Mice

Experiment I				
Group No.	Inoculum	No. of Mice	Mean % Parasitemia (day five)	Mean Survival Time (days)
I	Homogenate in Freund's adjuvant	7	16.9	15.4
II	Mouse erythrocytes in Freund's adjuvant	18	26.5	13.5
III	Buffered saline	18	29.9	11.8

The smaller number of animals in the groups receiving parasite homogenate are due to acute mortality (less than 16 hours after injection) presumably as a result of saponin toxicity. Separate experiments established that the minimum lethal dose of the lot of saponin used is between 100 and 1000 micrograms. On the basis of dilution during the washing procedure it was calculated that the animals would receive less than 1 microgram. This discrepancy is interpreted as being due to an interaction of saponin with the insoluble residue with release after injection.

Although differences are small the data suggested some possible protection by the parasite homogenate and to a lesser degree by the normal blood homogenate.

A second experiment was performed in which the parasites were washed six times instead of three. Injections were given on days 0 and 49 with challenge on day 56.

Table XXI summarizes the results of this experiment. Acute toxicity was again observed (eighteen animals were initially present in Group I). No evidence of protection was seen.

These studies indicate that a demonstrable immunity to P. berghei infections was not achieved by injections with this homogenate. Efforts are being shifted to other P. berghei hosts which offer greater promise as suitable components of model systems of immunity to malaria.

Table XXI

Effect of Homogenate on P. berghei Infections in Mice

Experiment II

Group No.	Inoculum	No. of Mice	Mean % Parasitemia (day three)	Mean Survival Time (days)
I	Homogenate in Freund's adjuvant	8	9.1	13.1
II	Mouse erythrocytes in Freund's adjuvant	18	8.5	12.1
III	Buffered saline	18	6.1	13.6

12. Rickettsial Disease Investigations in Thailand.

Field trips were made to different regions which were representative of the variety of habitats encountered in the 4 physiographic provinces of Continental Thailand, i.e., the Northern Hills and Valleys of the Continental Highlands, the Upper and Bangkok Plain of the Central Valley, the Southeast Coast, and the Khorat Plateau. Studies were not made in the Western Mountains or in the Peninsular portions of Thailand. In each of the areas visited, animals were trapped and their ectoparasites were removed and identified. Serum was collected and attempts were made to recover R. tsutsugamushi from spleen and liver by inoculation of tissue suspensions intraperitoneally into white mice.

The recovery of strains of R. tsutsugamushi from small wild mammals indicates its probable endemicity throughout most of Continental Thailand (see Table XXII). Isolations were made from animals trapped in the Northwest, in the Khorat Plateau near the Laos border, in the Southeast coastal region and in the upper and lower portions of the Central Valley rice plains. Among the 4 genera of animals which have been identified as vertebrate hosts for R. tsutsugamushi are included 5 species of Rattus, Tupaia, Menetes and Bandicota. It is of interest that 2 strains of scrub typhus rickettsiae were obtained from Rattus rattus trapped in the outskirts of Bangkok.

Thus far, Leptotrombidium deliensis is the only chigger which has been shown to be infected with R. tsutsugamushi. Successful recoveries of strains of scrub typhus rickettsiae were made from 6 pools of L. deliensis larvae removed from rodents captured in Chong Mek near the Laos border, and from 1 pool of this species of chigger similarly collected near Chiang Mai in Northwest Thailand.

TABLE XXII

SUSPECTED MAMMALIAN HOSTS OF RICKETTSIA TSUTSUGAMUSHI IN THAILAND

ANIMAL SPECIES	NUMBER OF STRAINS RECOVERED IN DESIGNATED PHYSIOGRAPHIC REGION *						TOTALS
	Continental Highland Northern Hills and Valleys	Central Valley		Southeast Coast	Khorat Plateau		
		Upper Plain	Bangkok Plain				
<u>Tupaia glis</u>	1	4	1	1	5	12	
<u>Rattus rattus</u>	5	7	7	-	13	32	
<u>Rattus rajah</u>	-	-	-	-	21	21	
<u>Rattus berdmorei</u>	-	-	-	1	1	2	
<u>Rattus viviventer</u>	-	-	-	-	1	1	
<u>Rattus exulans</u>	1	-	-	-	-	1	
<u>Menetes berdmorei</u>	-	-	-	-	3	3	
<u>Bandicota indica</u>	1	-	-	-	-	1	
TOTALS	8	11	8	2	44	73	

* The Western Mountain region of the Continental Highlands and the Peninsula region of Thailand were not studied.

The first isolations of scrub typhus rickettsiae from human beings in Thailand were made last year. Strains were recovered from 4 farmers in the Chiang Mai region. Two of the illnesses occurred during July and the others in September. Scrub typhus in each of these patients was a mild, nondescript disease. The clinical features included only fever, chills and headache. Rash or eschar were not observed.

Although murine typhus is suspected of occurring in Thailand, virtually nothing is known about this disease. The results of the serological survey on human sera reported last year (see Table 3, Annual Progress Report, WRAIR, 1 July 1962-30 June 1963, p 620) showed that 4 of 50 specimens collected from border police stationed in northwest Thailand contained significant levels of typhus group complement-fixing antibodies. In November 1963, an agent belonging to the typhus group was isolated from flea-infested Rattus exulans trapped in the market of Chiang Rai, located in the northwest portion of Thailand. Studies to definitively identify this strain are currently in progress.

Serological evidence for the presence of a member of the spotted fever group of rickettsiae in Thailand was presented in the Annual Progress Report, WRAIR, 1 July 1962-30 June 1963, p 619. An agent, designated TT-118, was recovered in guinea pigs from a mixed pool of Ixodes and Rhipicephalus larvae removed from Rattus rattus trapped in the Chiang Mai region in November 1962. Initial studies to identify this rickettsia have shown closer antigenic relationships to fever boutonuse and Indian tick typhus than to other members of the group (see Table XXIII). Serum from guinea pigs convalescent from an infection with TT-118 fixed complement with 2 units of a specific rickettsial antigen prepared from Rickettsia conorii, but not with similar antigens from R. sibericus, R. rickettsii and R. akari.

The results of complement fixation tests on wild animal sera collected during the scrub typhus field studies, using spotted fever and typhus group soluble antigens and Q fever antigen are presented in Table XXIV. A total of 396 sera representative of 16 different species of animals collected during the period Oct 1962 to May 1963 have been examined. Antibodies to one or more of these rickettsioses were found in only 5 of the species tested, i.e., Rattus rajah, Rattus rattus, Tupaia glis, Bandicota sp., and Cannomys badius. However, animals with spotted fever and Q fever antibodies were present in each of the physiographic regions sampled. Typhus group antibodies were found only in Tupaia glis in all of the regions except the Central Plain where specimens from this species were not available for testing.

Efforts were made to assess the incidence of Q fever infection in the domestic animals in Thailand. Captain Thomas J. Keefe, VC, U.S. Component, SEATO Medical Research Laboratory, Bangkok, provided aliquots of sera from 392 cattle and buffalo, and 61 swine, collected

TABLE XXIII

PRELIMINARY SEROLOGICAL IDENTIFICATION OF TT-118

ANTISERUM Guinea Pig 4 units	COMPLEMENT FIXATION TEST RESULTS							
	SPECIFIC ANTIGENS						SOLUBLE ANTIGEN	
	F. Bout		STT		RMSF		RPOX	
	2	1	2	1	2	1	2	1
	Units		Units		Units		Units	
TT-118	+	*	-	-	-	-	-	-
F. boutonneuse	+	+	-	-	-	-	-	-
Indian Tick Typhus	+	+	-	-	-	-	-	-
Siberian Tick Typhus	+	-	+	+	-	-	-	-
Rocky Mt spotted fever	-	-	-	-	+	+	-	-
Rickettsialpox	-	-	-	-	-	-	+	+
Queensland Tick Typhus	-	-	-	-	-	-	-	-

* Significant fixation of complement in presence of indicated amount of antigen and 4 units of antiserum.

** No fixation of complement.

during the course of his investigative activities. Unfortunately, the bovine sera had marked anticomplementary activity which precluded the use of this serological system in the study. As soon as suspensions of Coxiella burnetii for use in agglutination tests can be prepared, this aspect of the investigation will be completed.

In February 1964, field and laboratory investigations in Thailand were temporarily suspended to permit Captain Vichai Sangkasuvana to come to the Department of Rickettsial Diseases, WRAIR, for a 5-6 month period of training in rickettsial research. During his tour of duty at WRAIR it is expected that Captain Vichai will extend his capabilities to work with other rickettsial agents, i.e., members of the spotted fever and typhus groups, and Q fever, as well as complete an antigenic analysis of the strains of R. tsutsugamushi recovered in Thailand from wild animals, chiggers and patients.

TABLE XXIV

SUMMARY OF RESULTS OF SEROLOGICAL SURVEY OF THAI WILD ANIMAL SERA
OCT 1962 - MAY 1963

ANIMALS	PHYSIOGRAPHIC REGION OF THAILAND												TOTALS							
	NORTHWEST				KHORAT PLATEAU				SOUTHEAST								CENTRAL PLAIN			
	No.	SF	Q	Typh	No.	SF	Q	Typh	No.	SF	Q	Typh					No.	SF	Q	Typh
	Sera	Pos	Pos	Pos	Sera	Pos	Pos	Pos	Sera	Pos	Pos	Pos	Sera	Pos	Pos	Pos	Sera	Pos	Pos	Pos
<u>Rattus rajah</u>	9	1	1		36	7			12	3	1						57	11	2	
<u>Rattus rattus</u> sp.	22	1			17				6								51	2	1	
<u>Tupaia glis</u>	8	1		1	28	8	4	3	2	2	2	1					38	11	6	5
<u>Bandicota</u> sp.	6	2	2		2												26	8	4	
<u>Cannomys badies</u>	2		1														2		1	
TOTALS	47	5	4	1	83	15	4	3	20	5	3	1					174	32	14	5
OTHER SPECIES	<u>M. berdmorei</u> -15				<u>M. berdmorei</u> -2				<u>M. berdmorei</u> -13											
TESTED WITH	<u>R. mulleri</u> - 1				<u>R. mulleri</u> -2															
NEGATIVE RESULTS	<u>Hylomys</u> sp. 2																			
	<u>C. erythraeus</u> -1																			
	<u>Hylomes</u> sp. - 1																			
	<u>C. caniceps</u> - 1																			
	<u>M. javanicus</u> - 1																			
	<u>R. whiteheadi</u> -1																			

Serological Study of Vietnamese Sera for Rickettsial Diseases.

A total of 170 sera collected during the course of a Health Survey of a Jarai community in the village of Boun Khan, South Vietnam by Dr. E. Voulgaropoulos and Major E.J. Feeley, MC, USA, were referred to the Department of Rickettsial Diseases, WRAIR, for serological study. The results of complement fixation tests employing spotted fever group and typhus group soluble antigens and Q fever antigen, as well as the results of indirect immunofluorescent tests for scrub typhus are presented (see Table XXV).

TABLE XXV

RESULTS OF SEROLOGICAL TESTS ON 170 VIETNAMESE HUMAN SERA

Antigens	Number ** Positive	COMPLEMENT FIXATION TEST *			
		Distribution of CF titers			
		1:4	1:8	1:16	≥1:32
Spotted Fever Group Soluble	17	3	4	4 ⁺	6 ⁺⁺
Typhus Group Soluble	8	1	1		6
Q Fever	1		1		

* 100 sera were negative at a 1:4 dilution and 47 specimens were unsatisfactory due to anticomplementary activity.

** Titers of 1:4 or greater are considered significant of prior infection.

+ One serum also titrated 1:8 with typhus and Q fever antigens and was entered in these other categories.

++ One serum also titrated 1:32 or greater with typhus antigen and has been included in this category.

Antigen	Number ** Positive	INDIRECT IMMUNOFLUORESCENT TESTS *			
		Distribution of IF titers			
		1:10	1:40	1:160	1:640
Scrub Typhus ⁺	8	11	2	5	1

* 151 sera were negative at a 1:10 dilution.

** Titers of 1:40 or greater are considered significant and titers of 1:10 suggestive of prior infection.

+ The Karp, Gilliam and Kato strains were employed as antigens. The IF titer recorded is the highest dilution of serum which gave 1+ fluorescence with one or more of the antigens.

Of the 170 sera examined, 47 were unsatisfactory for complement fixation tests owing to anticomplementary activity. Significant levels of complement-fixing antibodies to one or more of the rickettsioses were found in 23 of the remaining 123 specimens. Spotted fever group antibodies only, were found in 15 sera and typhus group antibodies only, in 6 specimens. One serum had spotted fever group and typhus group antibodies, and another had antibodies to the previously mentioned rickettsial antigens as well as Q fever. Complement fixation tests with specific rickettsial antigens to differentiate between epidemic and murine typhus will be carried out as soon as reliable reagents can be prepared.

All 170 specimens were tested with antigens prepared from the Karp, Kato and Gilliam strains of Rickettsia tsutsugamushi in the indirect immunofluorescent tests for scrub typhus. Titers of 1:40 or greater with one or more of the antigens, which are considered significant of prior infection, were found in 8 specimens. Eleven other sera reacted at a dilution of 1:10 which is only suggestive of previous scrub typhus infection.

Further analyses of the data will be undertaken when information about the age, sex and occupation distribution within the population sample is made available.

13. Leptospirosis.

To date, approximately 1,523 leptospiral cultures isolated in Malaysia have been submitted to WRAIR for culture typing. Six hundred and thirty-six cultures were submitted during the period of this report and included 70 strains isolated from soil and water in North Borneo. The strains were derived for the most part during the course of epidemiological studies on the infectiousness of soil and water under various climatic and ecologic conditions. (See Annual Progress Report, USAMRU, Kuala Lumpur, Malaysia.) The general procedure employed for the typing of strains has been described in the previous annual report. Preliminary culture typing has been completed on 1,053 isolates obtained in Malaya and on 58 strains isolated in North Borneo. Seventy-five different cross-agglutination patterns (presumably reflecting the same number of serotypes) have been disclosed in Malaya. The strains could be classified in 10 of the 13 recognized serogroups. Ten different types of reactions characteristic of three serogroups were disclosed in the typing of the North Borneo strains. The distributions of the isolates from Malaya and North Borneo, according to serogroup and serotype (tentatively identified by representative strain), are shown in Tables XXVI and XXVII respectively. Five types comprised approximately half the strains isolated in Malaya. Approximately 48% of the strains identified from North Borneo represented one type in the bataviae group. The serological findings will be correlated with epidemiological data.

Hamster inoculation technics were used in Malaysia to recover leptospiras from soil and water. The demonstration of leptospirosis with this technic is contingent on the recovery of organisms from dying animals. In the previous annual report, it was noted that partial or questionable reactions were obtained with approximately 18% of 95 hamsters at high risk to infection. Two animals had relatively high titer agglutinins. These findings seemingly pointed to a high percentage of missed infections. To clarify the significance of the low titer agglutinins, serological tests were conducted on 47 normal hamsters from the animal colony at USAMRU, Malaysia. Partial reactions were seen in 6 or 12% of the sera. The indication from these findings is that relatively few infections in experimental hamsters were missed.

Leptospiral strains isolated from 46 swine, 9 rats, one cow, one buffalo and one cat in Thailand were forwarded to WRAIR for culture typing. These isolates were obtained during the course of an epizootiological study of leptospirosis. (See Annual Progress Report, U. S. Army Component, SEATO.) Twenty-three of the strains have been typed to date. The following serogroup identifications were established: pomona serogroup - 15 swine strains; javanica serogroup - 6 rat strains and one cat strain; and autumnalis serogroup - one buffalo strain. These results confirm typing findings in the Thailand laboratory.

Eight strains of leptospira isolated from rats in South Vietnam were submitted to WRAIR for culture typing by Dr. Duong-hung-Mo, Institute Pasteur de Vietnam. All strains were identified to be members of the bataviae group.

Serological tests for leptospirosis were conducted on 190 sera obtained from an indigenous population group (Montagnards) located in the highlands of the central portion of South Vietnam (Phu Ban). The sera were obtained by Dr. E. Voulgaropoulos and submitted to the WRAIR via USAMRU, Malaysia. Employing a sensitized erythrocyte-lysis (SEL) serological procedure, the USAMRU laboratories found antibodies in 53% of the samples. This remarkable high prevalence of antibodies could not be affirmed by the results of microscopic agglutination (MA) tests (the standard reference test) conducted at the WRAIR. There was poor correlation between the SEL and MA test findings. Significant titers ranging from 1:100 to 1:400 reflecting previous exposure to leptospirosis were obtained in MA tests on 9.2% of the samples. Partial reactions at 1:100 dilution were obtained with the screening antigens of pathogenic types in an additional 10.2% of the sera. These reactions are considered to be significant by most of the WHO Leptospirosis laboratories. Another 10.8% of the sera had partial reactions against a biflexa (non-pathogenic type) antigen.

Table XXVI

Preliminary Classification of 1,053
Leptospiral Isolates from Malaya
(76 types)

Sero-group	Type	No. of Strains	Sero-group	Type	No. of Strains	Sero-group	Type	No. of Strains
icterohaemorrhagiae (226 str.)	54i M-1185 75-373 M-33 M-203 M-108 M-1205 M-99 M-112 M-368 M-563	130 46 26 7 5 4 3 2 1 1 1	pyrogenes (124 str.)	M-50 29 55-271 113-561 M-84 M-97 M-48 198-998 M-36 247 M-211 M-31 M-88	58 14 14 10 10 6 3 3 2 1 1 1	hebdomadis (102 str.)	Ham 61 M-786 256(2) M-441 31a 193-972	78 9 8 5 1 1
australis (171 str.)	H-85 267-1349 M-1086 6P-6 6P-62 M-392	142 9 9 7 2 2	canicola (121 str.)	M-23 M-53 257 M-200 M-627 M-302 M-932 M-9 M-933 M-51 M-172 M-18 M-1104 M-357 M-1004	24 21 20 15 9 7 7 6 4 2 2 1 1 1 1	autumnalis (84 str.)	13KKB Blythe M-511 M-826 M-758 M-359 7-360 M-7 M-12 M-239	33 24 2 7 8 4 2 2 1 1
bataviae (153 str.)	20-96 267 M-87 264-1333	92 36 13 12				javanica (11 str.)	1160 807 33SB 267-1348 M-107 M-904	5 2 1 1 1 1
grippo-typhosa (58 str.)	6P-58 M-56 14	24 23 11				pomona (3 str.)	514	3

Table XXVII

Preliminary Classification of 58 Leptospiral
Isolates from North Borneo

Serogroup	Type	No. of Strains
bataviae (44 str.)	20-96	24
	87	10
	267	5
autumnalis (14 str.)	511	7
	360	2
	Blythe	2
	M-826	1
	7	1
	M-877	1
ictero- haemorrhagiae	54i	5

The significance of the biflexa reactions is questionable. Positive reactions were distributed amongst multiple antigens. The findings with the MA test point to a high prevalence of multiple leptospirosis in the sample population. Further tests will be conducted to resolve the reasons for the differences between the MA and SEL test findings.

Serological tests for leptospirosis on paired sera from 89 Special Forces soldiers (submitted through Virus Department, WRAIR) were negative.

Further studies were conducted towards the development of the multivalent leptospiral bacterin for use in Southeast Asia and other areas of multiple leptospirosis. The polyvalent vaccine was prepared with 5 different leptospiral types in the autumnalis, grippotyphosa, pyrogenes, hebdomadis and bataviae serogroups, respectively. (See previous Annual Progress Report.) The antigenic potency of the polyvalent bacterin was tested in mature rabbits. In separate experiments, the individual formalized antigens comprising the bacterin and the pooled bacterin were evaluated. The monovalent bacterins contained 70×10^6 organisms and was administered s.c. in a 1 ml dose at 3 weekly intervals. The multivalent vaccine contained approximately 350×10^6 organisms and was administered similarly with a 2.0 ml dose. Animals given the individual strains developed low titer agglutinins ranging from 1:10 to 1:64 after the first inoculation. No demonstrable booster effect was noted after the second and third inoculations. The pooled bacterin provoked low titer agglutinins (1:10 to 1:16) against pyrogenes only.

In view of these findings, the antigen concentration in the pooled bacterin was increased three-fold and reexamined for antigenicity. Four rabbits were given 3 s.c. inoculations of 1.0, 1.0 and 2.0 ml of multivalent bacterin successively at 7 day intervals. Four additional rabbits were inoculated similarly with an aliquot of bacterin to which an adjuvant, sodium alginate, was added. No remarkable differences in the antigenic response in the 2 groups of rabbits were seen. The antigenic response of rabbits given the bacterin without adjuvant is summarized in Table XXVIII. There was no uniform response amongst rabbits to vaccination during a 42 day period of observation. Reactions were frequently transient and ranged from 1:10 to 1:40 in titer. Agglutinins against the 5 antigens of the bacterin were seen, in various combinations, in 3 of 4 rabbits. Among the 4 rabbits, one developed titers against all 5 antigens, one and 2 against various combinations of 4 and 3 antigens, respectively.

The prophylactic efficacy of the bacterin was tested in mature (100 - 120 gm) hamsters. Two groups of hamsters, each comprising 25 animals, were inoculated s.c. respectively, with 0.1 ml and 0.5 ml doses of pooled bacterin containing approximately 350×10^6 leptospiras per ml. Two doses were given 11 days apart. Thirty-two days

Table XXVIII

Agglutinin Response in Four Rabbits to Multivalent
Leptospiral Vaccine

Test Antigen	Number of Rabbits Positive ¹		
	Day 10	Day 21	Day 42
grippotyphosa	0	1	3
pyrogenes	3	2	2
autumnalis	0	0	3
hebdomadis	0	1	3
bataviae	2	2	0

¹ Bacterin given on days 1, 7 and 14, titers ranged from 1:10-1:40.

after the second inoculation, 5 animals each from the 2 groups were challenged with virulent cultures of the different strains used in the bacterin. Each challenge strain was also inoculated into 5 control hamsters. Challenge consisted of 1.0 ml of 10^{-2} dilution of 7 - 10 day old Fletcher's culture and was administered i.p. The selection of dose was based on previous experiments. The MLD of various cultures ranged from 10^{-2} to 10^{-3} . Blood cultures were made on the third and sixth days post challenge on one animal from each group of 5 animals. Animals dying during the course of experiment were cultured for leptospira. At 28 days post challenge all surviving animals were sacrificed and their kidneys were cultured for leptospira. A summary of findings is shown in Table XXIX. When given at 0.5 ml dose, bacterin protected all hamsters against lethal challenge with grippytyphosa, pyrogenes and hebdomadis serotypes and all but one or 2 animals in each group of 5 challenged with autumnalis and bataviae. Protection against lethal infection with grippytyphosa was also afforded hamsters vaccinated with a five-fold lower dosage. In the lower bacterin dosage group, protection against other serotypes was evident by the difference in ratio of deaths and time of deaths in vaccinated animals and controls. In neither group did vaccination ensure complete protection against infection.

14. Melioidosis.

Studies on melioidosis were initiated with the USAMRU in Malaysia. (See Annual Progress Report, USAMRU, Malaysia.) First efforts were directed towards the location of natural foci of infection, the finding of cases amongst patients with undiagnosed pulmonary infections, and the collection of sera from population groups with possible risks to infection. Employing hamster inoculation technics in conjunction with newly developed cultural procedures, foci of infection have been located principally in stagnant mining pools but also in rice fields and in rivers in and about the city of Kuala Lumpur. To date, 40 of 70 cultures isolated from soil and water and forwarded to the WRAIR have been verified to be Pseudomonas pseudomallei. Bacteriological examination of sputum samples (at USAMRU) and CF tests on sera from chest clinic patients have been unproductive thus far. CF tests for melioidosis were also conducted on 133 single paired sera from 91 Malayan Federation engineer soldiers and on paired sera from 84 S.F. soldiers (submitted via Virus Department, WRAIR). In the former series, positive titers were obtained in 7 persons, including 5 from whom paired sera were obtained 7 months apart. Changes in titer were seen in 3 and stationary titers in 2 persons. Significant CF titers were obtained only in the second serum samples from 5 of 84 S.F. soldiers.

The CF test for melioidosis presumably detects antibodies elicited in recent infections occurring 3 to 6 months previously.

Table XXIX

Results of Protection Studies of Multivalent
Leptospiral Bacterin in Mature Hamsters

Bacterin Dose	Challenge ¹	Vaccinates		Controls		Vaccinates	
		No. Dead	Av. Day	No. Dead	Av. Day	Lepto- spiremia ²	Lepto- spiremia ³
		No. Tested	Death	No. Tested	Death		No. Pos. No. Tested
.5 ml two doses 11 days apart	autum.	1/5	13.0	5/5	6.4	-	0/4
	gripp.	0/5		4/5	9.7	+	0/5
	pyrog.	0/5		4/5	11.0	+	1/5
	hebd.	0/5		4/5	8.0	+	1/5
	batav.	2/5	9.5	5/5	7.6	+	1/3
.1 ml two doses 11 days apart	autum.	3/5	14.0			+	1/2
	gripp.	0/5				-	1/5
	pyrog.	3/5	15.0			+	1/2
	hebd.	2/5	6.5			+	0/3
	batav.	2/5	9.5			+	2/3

¹ 1.0 ml of 10⁻² dilution of culture given 32 days after third vaccination.

² One animal tested on third and sixth day post-challenge.

³ All three surviving control animals positive.

The need for a more sensitive serological tool for epidemiological surveys prompted an evaluation of a hemagglutination test procedure. The antigen employed for this purpose was prepared according to methods described by Olds and Lewis (Austr. Vet. J., 56: 253, 1954), and essentially consisted of a sterile filtrate of 14 day old culture of P. pseudomallei cultivated in a protein-free fluid medium. Sera employed in initial evaluation of HA tests were antisera against various strains of P. pseudomallei and Actinobacillus mallei prepared in rabbits, and also CF positive and negative human sera obtained from Malaysia. The general procedures of Stavitsky (J. Immunol. 72: 360, 1954) were followed in conduct of HA tests. Initial studies were made to determine the optimum concentration of antigen and sheep red blood cells and the optimum temperature and time of incubation for conducting the test. Based on these studies, a 2.5% suspension of washed red blood cells was sensitized by treatment with an equal volume of antigen, then incubated at 37° C for one hour, centrifuged, washed 3 times and resuspended to the original concentration (2.5%). To 0.5 ml amounts of varying dilutions of the test serums (previously adsorbed with sheep erythrocytes) was added 0.05 ml amounts of suspension sensitized red blood cells. The antigen antibody mixtures were then incubated 2 hours at room temperature and examined for hemagglutination. Titers with 3 anti-pseudomallei sera ranged from 1:10,240 to 1:20,480, titers with 2 anti-mallei sera were 1:1280. No reactions were obtained with the following hyperimmune rabbit anti-serum: Pseudomonas aeruginosa, Listeria monocytogenes, Pasteurella tularensis, Brucella abortus, Salmonella (Dublin), Leptospira (hebdomadis). The SEL test was applied to 133 sera from 91 Malaysian engineers. Reactions ranging in titer from 1:5 to 1:40 were found in 39 samples from 34 soldiers. All samples positive on the CF test elicited reactions in HA tests. The distribution of maximum titers amongst Federation engineers was as follows: 1:5 - 9; 1:10 - 20; 1:20 - 2; and 1:40 - 3. Positive reactions were obtained from 12 individuals from whom paired sera taken approximately 7 months apart were available. In only 4 of the 12 pairs were reactions obtained in both samples and in only one of these cases was there a demonstrable rise in titer (1:20 to 1:40). In 8 other paired sera, the initial serum samples were positive at dilutions of 1:5 to 1:10; whereas, later samples were negative. The distribution of HA positive reactions among the 9 sera from 7 CF positive soldiers were as follows: 1:5 - 3; 1:10 - 3; 1:20 - 1; and 1:40 - 2. The significance of the low titer reactions will be further evaluated in tests conducted with normal human sera obtained in the United States.

It was found that tanned or formalin-treated sheep red blood cells could be sensitized with the culture filtrate antigen to react specifically with anti-pseudomallei serum. Titers were two to four-fold lower employing tanned cells; with formalin-treated cells, titers were comparable to those seen with untreated cells.

Summary and Conclusions:

1. Using the hemagglutination and complement fixation tests on wild and commensal rodent sera with a purified Pasteurella pestis Fraction 1 antigen, several active plague foci could be differentiated. These procedures carefully used and evaluated should permit reliable, focal evaluation of critical areas for estimation of a plague threat and the effectiveness of control programs.

2. Development and testing of a new transport medium for the collection and shipment of clinical specimens from epidemic sources to a central laboratory facility is described. Recovery of Shigella spp. for up to 45 days and Salmonella spp. for 62 days from rectal swabs is noted. P. pestis has remained viable in swabs from clinical material for seven months. Widespread use of this medium should permit greater bacteriologic support of field investigations.

3. Qualitative and quantitative studies were carried out on 203 specimens from 131 traumatic wounds. Degrees of infection could be related directly to (a) the type of wound and (b) the wounding agent, but not to the anatomical location of the wound. Bacteria most frequently isolated from fresh wounds were staphylococci, fecal streptococci and coliform in that order. The bacteria of seriously infected wounds were staphylococci and pseudomads, both of which were considered to be resident organisms of the hospitals involved. While Clostridia were present in 16 wounds in relatively large numbers, and persisted for days, there was no evidence of clinical gas gangrene.

4. A modified fluorescent antibody test was developed for the laboratory diagnosis of human malaria. This technic was evaluated with sera from individuals with bacterial, mycotic and parasitic infections. Minute amounts of dried blood specimens obtained in endemic areas on absorbent paper were tested satisfactorily for malarial antibodies. Although broad cross reactivity was observed between Plasmodium vivax and Plasmodium falciparum a greater degree of sensitivity and higher titers were observed in the homologous systems.

5. Preliminary experiments on the physiological pathology of Plasmodium berghei infections in mice indicated that infected animals had significantly lower values of chloride, glucose, alkaline phosphatase, phosphorus and total protein. In infected mice significantly increased SGOT transaminase and carbon dioxide values were observed. These studies are being continued and extended to include investigations with human volunteers.

6. A method for the direct enumeration of P. berghei parasites in the blood of infected animals was developed. Evaluation of this technic indicated that in addition to avoiding the necessity for two separate determinations with subsequent calculation of the parasite concentration, this technic gives more reproducible results than the determination of the per cent parasitemia from conventional thin blood smears.

7. Immunodiffusion analysis of P. berghei was performed. The agar gel diffusion technic revealed a minimum of five different precipitin bands attributable to the parasitic antigen. When subjected to qualitative chemical tests for further characterization, two of the components gave positive reactions for lipid components. These studies also revealed that a soluble extract of P. berghei contains a number of materials of host origin among which hemoglobin is quantitatively the most important.

8. Studies of the relationship of the size of inoculum and infections with P. berghei in mice reveal that none of the animals receiving less than 10,000 parasites developed a detectable parasitemia. The LD 50 of mice under these conditions was approximately 70,000 parasites.

9. A search for suitable hosts for P. berghei indicated that the muskrat is the most susceptible to this infection of all animals tested. In this animal death ensued after a parasitemia reaching 90%. Severe infections were also produced in gerbils. Moderate infections were obtained repeatedly in splenectomized Rhesus monkeys.

10. The effect of irradiation on the infectivity of P. berghei was studied. A dose of 13 Kr is sufficient to prevent the obvious parasitemias and death of the mice. No demonstrable immunity developed in mice following injection with parasites attenuated by ionizing radiation.

11. Attempts to induce immunity to P. berghei in mice following injection of parasite homogenates have been so far unsuccessful.

12. Data collected during field and laboratory investigations in the past year have contributed significantly to an understanding of the distribution and ecology of scrub typhus in Thailand. Recovery of strains from wild animals indicates the probable widespread endemicity of Rickettsia tsutsugamushi throughout most of Continental Thailand. Eight species of small mammals included among 4 genera have been implicated as possible vertebrate reservoirs. Thus far, only Leptotrombidium deliense has been incriminated as vector. The mildness

and lack of specific manifestations of the illnesses of 4 scrub typhus patients diagnosed by recovery of the causative agent may explain why the disease is not recognized by physicians in Thailand. A total of 84 isolations of *R. tsutsugamushi* were made from animals, chiggers and human beings, and an antigenic analysis of these strains is currently in progress. A sero-epidemiological study of sera from small wild animals trapped in the various physiographic provinces of Continental Thailand revealed that Q fever and some member of the spotted fever group of rickettsiae are widely enzootic. Diseases caused by these agents in man have not been recognized. Bovine sera have been obtained from Thailand and an assessment of the incidence of Q fever infection of these domestic animals will be made in the near future. A strain belonging to the spotted fever group was isolated from ticks and another agent believed to be murine typhus was recovered from a rat. Definitive identification studies of these rickettsiae are in progress.

The results of complement fixation tests on 123 sera obtained from inhabitants of a Jarai community in the central portion of South Vietnam showed that 15% contained spotted fever group antibodies, 6.5% had typhus group antibodies, and 1% had demonstrable Q fever antibodies. Utilizing the indirect immunofluorescent test, scrub typhus antibodies were found in about 5% of the specimens.

13. The number of leptospiral strains isolated in Malaysia during the course of epidemiological studies on factors affecting the leptospiral contamination of environmental foci of infection now total 1523. Approximately 636 strains were isolated during the past year including 70 strains from N. Borneo. Preliminary culture typing tests have now been completed on 1053 isolates obtained in Malaya and 58 isolates from North Borneo. The presence of approximately 75 different serotypes has been revealed in Malaya. Ten different types have been disclosed amongst strains obtained from N. Borneo. Approximately half of the Borneo strains belonged to the same type member of the bataviae group. That North Borneo, like Malaya, comprises epidemic and endemic areas of multiple leptospirosis is indicated from cultural and serological findings. The sensitivity of hamster inoculation technics to recover leptospiras from soil and water -- the principal procedure used in epidemiological studies in Malaysia -- was further evaluated by comparative serological studies on normal hamsters and experimental hamsters at high risk of infection but with no manifest signs of disease. Contrary to initial indications, the percentage of missed infections in experimental hamsters used in Malaysia is relatively low -- probably less than 10 per cent.

Culture typing tests were also conducted on 23 of 58 strains submitted from Thailand. All of 15 isolates from swine were identified to be members of the pomona serogroup; isolates from 6 rodents and 1 cat were identified to belong to javanica serogroup and an autumnalis type was isolated from a buffalo. Within the limitations of the conduct of epidemiological studies in Thailand, the multiplicity of types characteristic of the Malayan leptospirosis has not been apparent in Thailand.

Isolates from rats in South Viet-Nam were identified as members of the bataviae group. A high prevalence of significant agglutinins (10-20%) was revealed in an indigenous population group in South Viet-Nam. Occurrence of multiple serotype infections was indicated. In the same population group, the prevalence of antibodies was 53% when tested with a sensitized erythrocyte lysis procedure in Malaya. Most of the positive reactions are probably non-specific. Serological tests for leptospirosis on paired sera from 89 Special Forces troops were negative.

An experimental multivalent bacterin developed for use in areas of multiple leptospirosis was further evaluated. The antigenicity of the preparation was relatively poor when measured by the agglutinin response of inoculated rabbits. Titers were in the order of 1:10 to 1:40. However, the bacterin afforded mature hamsters protection against death when challenged with lethal doses of cultures of 5 diverse serotypes. It did not, however, ensure immunity to infection. Further attempts are being made to increase the antigenicity of the bacterin before trials in larger animals are initiated.

14. Studies on melioidosis were initiated in Malaya with the USAMRU in Kuala Lumpur. Employing hamster inoculation technics in conjunction with new cultural procedures, attempts to locate water and soil foci of infections have been successful. The finding of undiagnosed cases of disease amongst chest clinic patients has not been successful to date. Evidence of the occurrence of inapparent infections in this area was again found by the disclosure of CF antibodies in 7 of 91 Malayan engineer troops and in 5 of 84 Special Forces soldiers. In the latter group, serum samples were paired and positive reactions developed in the second serum samples only.

A more sensitive serological procedure for melioidosis was sought through the use of hemagglutination test procedures. A technic for conduct of the test was developed. In trials with hyper-immune rabbit serum, specific high titer reactions in the order of 1:10,240 to 1:20,480 were obtained with anti-pseudomallei serum and titers of 1:1,280 were elicited with antiserum against the related glanders organisms. No reactions were obtained with P. aeruginosa, L. monocytogenes, P. tularensis, B. abortus and Salmonella and Leptospira antisera. The hemagglutination test was found to be more sensitive than the CF test in eliciting reactions in the series of sera from Malayan engineers. Reactions ranging in titer from 1:5 to 1:40 were obtained in 34 soldiers including the 7 with positive CF findings. The specificity of these reactions will be studied further.

ARMY RESEARCH TASK REPORT		REPORTS CONTROL SYMBOL CSCRD-6(R2)
ACCESSION NUMBER 36217	PROJECT, TASK, OR SUBTASK NO. 3AO25601A8160151	
1. REQUESTING AGENCY The Army Medical Service Office of The Surgeon General Washington, D. C., 20315	2. FUNDING AGENCY Army Medical R&D Command Office of The Surgeon General Washington, D. C., 20315	
3. CONTRACTING AGENCY NA	4. CONTRACTOR AND/OR GOV'T LABORATORY A Walter Reed Army Inst of Rsch Walter Reed Army Medical Center Washington, D. C., 20012 723-1000, Ext 3552	
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Knoblock, Edward C., Lt Col, MSC, Dept Clinical Chemistry Division Biochemistry, WRAIR, WRAMC, Wash. D. C. 20012 576-3528 or Interdepartmental Code 198, Ext 3528 See Cont. Sheet		
6. TITLE OF: PROJECT <input checked="" type="checkbox"/> TASK <input type="checkbox"/> SUBTASK <input checked="" type="checkbox"/> Military medical materiel (U)		
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964		
8. RESUME: (U) Work has continued for development of laboratory equipment items designed to alleviate logistical burden for laboratory support of field operations. Prototypes of laboratory equipment include a centrifuge, titration unit, spectrophotometer, and pH meter. All instruments operate mechanically or from self-contained, rechargeable battery power sources. Work is continuing on evaluation and selection of the optimum biochemistry procedures to be packaged for quick use in the field. Final prototypes are soon to be delivered and a field testing exercise has been planned for late 1964. Two prototypes of water purification systems for preparation of pyrogen-free, sterile distilled water have been developed and tested. Water produced by this compression still from highly contaminated source water was consistently found to be pyrogen-free and sterile and to meet the requirements of US Pharmacopoeia for water for injection. Applications of gas chromatography in analytical toxicology have provided methods of identification of drugs where no other specific procedure exists. Such drugs include meprobamate and glutathamide. Sensitivity is sufficient to detect and identify 5-10 micrograms of the drug.		
9. KEY WORDS Equipment, water purification, gas chromatography, drug identification, laboratory procedures.		
10. SUPPORTING PROJECTS Not Applicable		
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet	12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet	
<div style="display: flex; justify-content: space-between;"> DA FORM 1309R 1 June 63 PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of ____ </div>		

ACCESSION NO.

36217

ARMY RESEARCH TASK REPORT

13. PROJECT, TASK OR
SUBTASK NUMBER

11	22	23 24	25 26	27 28	29
3 A 0 2 5 6 0 1 A 8 1 6	0 1	5 1			

14. DATE OF REPORT (30-33)

30	33	34
0 6 6 4	4	

15. SECURITY OF WORK (34)

16. TYPE OF REPORT

35	36	47 48	49 50	51	52	55
1						

17. SCIENTIFIC FIELD

a. Topical Classific. (56-61)
b. Functional Class (62-64)

56	61	62	64
0 1 0 7 0 0			

18. OSD CLASSIFICATION
(65-66)

19. R&D CATEGORY (67)

65 66	67
A R	2

20. CONTRACT NUMBER

11 12	13 14	15	17	18	21	22	26	27
D A								

21. GRANT NUMBER

28 29	30	33	34 35	36	38	39 40	41	45	46
D A							G		

22. ESTIMATED COMPLET.
DATES

47	51	52	56	57	61	62	66	67	71
1 0 0 N T	2			3		4		5	

23. PRIORITY (11-14)

24. PROGRAM ELEMENT
(15-26)

11	14	15	26
	1	6 • 0 1 • 5 6 • 0 1 • 1	

25. CMR&D CODES

27	29	30	32	33	35
N / A					

26. CDOG REFERENCE

a. Paragraph No. (36-44)
b. Functional Group (45)

36	39	40	41 42	43 44	45
					6

27. FUNDING

a. Est. Total Cost (11-15)
b. % Spent Intern. (16-18)
" " Extern. (19-21)
c. Total Obligation (22-26)
d. Progrmd. Cur. FY (27-33)
e. " " " +1 (34-40)
f. " " " +2 (41-47)
g. " " " +3 (48-54)
h. " " " +4 (55-61)
i. " " " +5 (62-68)
j. " " " +6 (69-75)
k. Total Man Years of
Effort (76-78)

11	15	16	18	19	21	22	26
	9 2	1 0 0	2				7 0
27 28	29	33	34 35	36	40		
6 4		7 0	6 5		1 4 0		
41 42	43	47	48 49	50	54		
6 6		1 4 7	6 7		1 5 4		
55 56	57	61	62 63	64	68		
6 8		1 6 2	6 9		1 7 0		
69 70	71	75	76	78			
7 0		1 7 9		1 0			

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

- (A) Maher, J. R. , Maj, MSC, Dept of Clinical Chemistry,
Div of Biochem, WRAIR, WRAMC, Washington, D. C. , 20012
576-3528 or Interdepartmental Code 198, Ext 3528 49
- (A) Kazyak, Leo, B. S. , Dept of Clinical Chemistry,
Div of Biochem, WRAIR, WRAMC, Washington, D. C. 20012
576-3528 or Interdepartmental Code 198, Ext 3528 49
- (A) de Baare, Larissa, M. D. , Dept of Clinical Chemistry,
Div of Biochem, WRAIR, WRAMC, Washington, D. C. , 20012
576-3528 or Interdepartmental Code 198, Ext 3528 49
- (A) Kenton, Edith, B. A. , Dept of Clinical Chemistry,
Div of Biochem, WRAIR, WRAMC, Washington, D. C. 20012
576-3528 or Interdepartmental Code 198, Ext 3528 49

REPORTS. Annual Progress Report, Walter Reed Army Institute
of Research, 1 July 1963 - 30 June 1964.

PUBLICATIONS.

Kazyak, L. , Knoblock, E. C. : Applications of Gas Chromatography to
Analytical Toxicology. Anal. Chem. , 35:1448-52, 1963.

ANNUAL PROGRESS REPORT

Project No. 3A025601A8160 Title: Military Medical Materiel

Task No. 01 Title: Military Medical Materiel

Subtask No: 51 Title: Military medical materiel

Description: This task was designed to evaluate a number of problems involving medical laboratory operations under field conditions and to attempt a solution to some problems which slow progress, impede flexibility, require excessive logistical support or are currently not satisfactory for military operations outside fixed installations. The principal areas included a) laboratory equipment for biochemical support; b) improved methods of producing pure water in the field; c) more rapid toxicological identifications.

Progress: Biochemical Analysis.

In an effort to develop an ultramicro analytical system suitable for either the fixed military hospital or field clinical laboratory, the Beckman Spinco Ultramicro Assembly is being used as the basic unit to undergo required adaptation. The Beckman Spinco Spectrocolorimeter, titrator, centrifuge and auxiliary equipment generally proved superior to comparable equipment available from other manufacturers.

An intensive investigation of current ultramicro techniques and procedures was performed. An important phase of this investigation was the study of the methods utilized by M. C. Sanz, Director of Clinical Chemistry, Pediatric Hospital, Geneva, Switzerland, who is a leading investigator in the development of ultramicro clinical techniques. A member of this department, L. de Baare, M. D., spent a period of temporary duty in Dr. Sanz laboratories to study details of his system and to observe his methods of training technicians.

The information obtained from this investigation is being evaluated and procedures are being developed which will be most suitable in providing a valuable diagnostic tool in military medicine. The use of specialized pieces of equipment with these procedures is also being studied.

The use of plastic pipettes, tubes and reagent bottles and the compact nature of essential equipment indicate that an ultramicro analytical system has many advantages over a macro system in mobile military field laboratories.

Progress: Water Purification

The provision of potable water for his men and animals historically has been the leading logistical concern of the Army Commander in the field. Food and weaponry are perhaps comparable with water in essentiality for combat, but only water requires uninterrupted supply.

Today it is assumed that all water to be used by troops must be treated by purification processes before it can safely be consumed.

With the advent of chlorination, the army found a readily adaptable system which could be used by the Corps of Engineers for producing water for troop use, and which could be provided in various forms to meet the needs of the individual by canteen treatment or the larger units by establishment of water points. No doubt this system works well under many conditions but certain diseases such as amoebic dysentery of the warm climates are highly resistant to chlorine. The advent of possible contamination by radioactive materials likewise poses a problem which has caused considerable concern and which in itself requires such special treatment procedures as distillation or ion exchange for reduction of these ions to an acceptable level for drinking water.

The Army Medical Department has even greater problems in providing a "USP Water for Injection" for use in preparation of intravenous solutions and the various medicines requiring a pyrogen-free, sterile water of low solids content-free of noxious materials. The experience of the past has been to provide vast quantities of such water by contract from commercial sources. These water solutions are packaged in fragile glass bottles, shipped long distances with attendant breakage, and stored under conditions which would, it is hoped, be compatible to longest possible shelf life of the aqueous solution. Each of these considerations has posed a continuing problem of considerable magnitude to the logisticians. On analysis each factor can be considerably alleviated if the water component need not be transported to the point of use. The principal bulk of any five percent solution is water. If this is eliminated and the ingredient shipped in the dry form where chemical stability is much better than in solution, there results a weight reduction by a factor of at least 20; which would be even greater if a suitable plastic bag were used as a container to replace glass.

By the single consideration of packaging chemicals in the desired weight into non-breakable containers which can at time of use be brought to the desired volume by a suitable water supply, the logistics attending resupply can be remarkably decreased. Not only is cubage and weight grossly reduced, but

breakage is essentially eliminated.

Recent research by the Army Medical Department has produced a system for making pyrogen-free, sterile water of very low solids content from a wide variety of water sources. The apparatus under investigation is a compression still which was developed under contract with Beckman Instruments, Fullerton, California. The first prototype has been delivered for user testing. Preliminary tests with this unit indicate that a very high quality of water is produced which is free of both bacteria and pyrogenic substances. The solids content is well below the requirements established for water for intravenous use. This unit produces approximately 200 liters of water in 24 hours, is rugged and compact in design, is simple to operate, and requires no plumbing attachments. It operates from 110 volt current with high efficiency. Although the preliminary model weighs approximately 140 pounds, many obvious ways are apparent to trim this model to a weight of less than 100 pounds without loss of operating efficiency. Other factors indicate increased output to be easily attainable to meet such requirements.

With design of a satisfactory collection and distribution system it is entirely possible that the central hospital supply in the Field Hospital of the future can satisfactorily reconstitute parenteral fluids on site at great logistical saving. Any of the facilities (laboratory, pharmacy, etc) will find similar advantages. It is further conceivable that larger units of similar design may be used to overcome many of the known problems encountered in furnishing potable water for troop use.

Progress: Toxicological Analyses.

Since chromatography and spectrophotometry afford very effective means for the analysis of compounds of toxicological interest, a study has been under way to utilize these techniques to determine those poisonous plant substances which tend to escape detection by ordinary screening procedures in toxicology. Compounds such as the cardiac glycosides, toxic polypeptides, and toxalbumins have come to the fore due to their great toxicity and ready availability in tropical areas. Methods to determine these substances rapidly and accurately in biological specimens are the main preoccupation of this task at present.

Whenever possible ultraviolet spectra have been determined, e. g. amanitotoxins (polypeptides) and abrin (toxalbumin). Infra-red spectra are recorded also, but this information is limited since most of the compounds under study are water soluble and therefore difficult to prepare for infrared determination. Moreover, infrared spectrophotometry is comparatively insensitive to the small concentrations of these compounds which may be recovered from blood and urine specimens.

The high boiling, high molecular weight characteristics of glycosides, and polypeptides tend to preclude analysis by gas chromatography although some effects to produce volatile derivatives have produced encouraging results. Certain digitalis glycosides can be analyzed in this manner (e. g. Digitoxigenin, Tigogenin), but attempts to apply the technique to serum-glycoside determinations have not been successful to date.

Thin-layer-chromatography is being substituted for paper chromatographic techniques which were employed in the early phases of this investigation. Some of the advantages have been a significant reduction in the time required for analysis and greater sensitivity. When applications of the thin-layer-chromatography technique have been completed on biological specimens which contain compounds under study, it is hoped that a simple, definitive scheme of analysis can be derived which will provide rapid, reliable results comparable to those obtained by gas chromatographic applications to drug analyses.

Ultraviolet spectra and gas chromatographic data on new drugs continue to be investigated consistent with a policy of compiling such information for distribution to Army Area Laboratories engaged in toxicological analyses. The purpose of this endeavor is to help update files and incorporate procedural changes when necessary.

Analytical toxicology support is being rendered the Department of Metabolism on studies of renal clearances of drugs after intraperitoneal dialysis on cases of drug intoxication admitted to Walter Reed General Hospital. The effectiveness of such agents as THAM (Tris(hydroxymethyl) amino-methane) in the dialysis procedure is presently being evaluated by the Department of Metabolism, and the concern of this laboratory is to determine the drug concentrations in urine, blood, and dialysate specimens so that drug clearance rates can be estimated.

Summary and Conclusions:

Evaluation of ultra-micro biochemistry procedures has established that many are very satisfactory for routine clinical work. The adoption of such techniques offers a large advantage logistically for military support.

A water distillation system has been developed which will produce pyrogen-free, sterile water from highly contaminated source water. This system utilizes the vapor compression principles and provides a portable unit which requires minimal utility connections, low power requirements, and a high level of confidence in operation.

Advances in instrumentation now enable more accurate and rapid identification of many compounds of toxicological significance. Included in this category are the toxalbumins, toxic polypeptides, and many of the glycosides which cannot be analyzed quantitatively in biological specimens. Utilizing the most recent techniques in thin-layer and gas chromatography, in conjunction with infrared and ultraviolet spectrophotometry, the present investigation is directed toward a simple, reliable scheme of detection and quantitation of these compounds in blood, urine, and tissue.

ARMY RESEARCH TASK REPORT				REPORTS CONTROL SYMBOL CSCRD-6(R2)	
ACCESSION NUMBER 37354			PROJECT, TASK, OR SUBTASK NO. RD41-61 c 03.008		
1. REQUESTING AGENCY US Army Combat Development Command, Nuclear Group Fort Bliss, Texas			2. FUNDING AGENCY Defense Atomic Support Agency Washington, D. C. 20315		
3. CONTRACTING AGENCY <div style="text-align: center;">NA</div>			4. CONTRACTOR AND/OR GOV'T LABORATORY A Walter Reed Army Inst of Research Walter Reed Army Medical Center Washington, D. C. 20012 723-1000 Ext 3552 <div style="text-align: right; border: 1px solid black; width: 20px; float: right;">49</div>		
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Sharp, J. C., Capt, MSC, Ph.D., Department of Experimental Psychology, Division of Neuropsychiatry, WRAIR, WRAMC, Washington, Sheet D. C. 20012 576-5262 or Interdepartmental Code 198, Ext 5262. See Cont/ <div style="text-align: right; border: 1px solid black; width: 20px; float: right;">49</div>					
6. TITLE OF: PROJECT <input type="checkbox"/> TASK <input type="checkbox"/> The Effects of Radiation on Sensory and Motor Functions SUBTASK <input checked="" type="checkbox"/> of Rhesus Monkeys(U)					
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964					
8. RESUME' (U) The completion of the first phase of this project yielded time-dose relationships that were necessary for the design of the second phase. The necessary methodologies for this second phase, i.e., the assessment of sensory capacities and motor coordination have been worked out and base-lines obtained on various monkeys. The data acquisition and reduction is being handled by a special high speed paper punch which is compatible with present computer facilities. Arrangements for a study of the effects on behavior of pulsed and mixed radiations compared to X-rays delivered at lower rates have been made with AFFRI. Studies of the effects of sublethal X-rays on vision, audition and emotional behavior have been completed, using rats as experimental animals. In addition to methodology development it was found that critical flicker fusion (vision) is less radiosensitive than tone discrimination, (auditory) and that emotional behavior is very radiosensitive.					
9. KEY WORDS Radiation, incapacitation, behavior, rhesus monkey, motor function, sensory function, X-ray effects					
10. SUPPORTING PROJECTS not applicable					
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet			12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet		
<div style="display: flex; justify-content: space-between;"> <div>DA FORM 1309R 1 June 63</div> <div>PREVIOUS EDITIONS ARE OBSOLETE</div> <div>PAGE 1 of ____</div> </div>					

ACCESSION NO. 37354		ARMY RESEARCH TASK REPORT	
Card "C"	13. PROJECT, TASK OR SUBTASK NUMBER	11 22 23 24 25 26 27 28 29	R D 4 1 - 6 1 c 0 3 . 0 0 8
	14. DATE OF REPORT (30-33)	30 33 34	0 6 6 4 4
	15. SECURITY OF WORK (34)		
	16. TYPE OF REPORT	35 36 47 48 49 50 51 52 55	3 0 6 6 3
Card "D"	17. SCIENTIFIC FIELD a. Topical Classific. (56-61) b. Functional Class (62-64)	56 61 62 64	0 5 0 1 0 0
	18. OSD CLASSIFICATION (65-66)	65 66 67	A R 1
	19. R&D CATEGORY (67)		
	20. CONTRACT NUMBER	11 12 13 14 15 17 18 21 22 26 27	D A
Card "E"	21. GRANT NUMBER	28 29 30 33 34 35 36 38 39 40 41 45 46	D A G
	22. ESTIMATED COMPLET. DATES	47 51 52 56 57 61 62 66 67 71	1 0 6 6 9 2 3 4 5
	23. PRIORITY (11-14)	11 14 15 26	
	24. PROGRAM ELEMENT (15-26)		
Card "F"	25. CMR&D CODES	27 29 30 32 33 35	
	26. CDOG REFERENCE a. Paragraph No. (36-44) b. Functional Group (45)	36 39 40 41 42 43 44 45	0
	27. FUNDING	11 15 16 18 19 21 22 26	
	a. Est. Total Cost (11-15) b. % Spent Intern. (16-18) " " Extern. (19-21) c. Total Obligation (22-26) d. Progrmd. Cur. FY (27-33) e. " " " +1 (34-40) f. " " " +2 (41-47) g. " " " +3 (48-54) h. " " " +4 (55-61) i. " " " +5 (62-68) j. " " " +6 (69-75) k. Total Man Years of Effort (76-78)	27 28 29 33 34 35 36 40 41 42 43 47 48 49 50 54 55 56 57 61 62 63 64 68 69 70 71 75 76 78	5 7 1 0 0 2 1 7 4 6 4 5 7 5 6 6 7 5 6 8 8 0 6 9 8 0 3

REPORTS. Annual Progress Report, Walter Reed Army Institute of Research, 1 July 1963 - 30 June 1964.

Sharp, J. C.: Ionizing radiation and primate behavior. Tenth V.A. Conference on Psychiatric Chemotherapy, Kansas City, Missouri, 1963.

Murphy, G. P., and Sharp, J. C. The effect of radiation and cold immersion upon experimental urolithiasis, renal function, and morphology. Invest. Urology., 1: 282-297, 1963.

Sharp, J. C. and Daoust, D. L. The effects of acute and massive doses of x-irradiation on primate behavior. Walter Reed Army Institute of Research, Division of Neuropsychiatry, Tech Rept #3, January, 1964.

ANNUAL PROGRESS REPORT

Project No. RD41-61

Title: Biomedical (NWER) (DASA)

Subtask No. 03.008

Title: The Effects of Radiation on
Sensory and Motor Functions
of Rhesus Monkeys

Description: The time to incapacitation following exposure to lethal doses of radiation has not been previously determined though this information is seemingly important to both offensive and defensive planning. The techniques of assessing changes in sensory and motor functions of rhesus monkey (*macaca mulata*) which were developed for other purposes have been adapted to bear on this problem. Several studies using rats have been completed. These studies explored the problems of measuring the effects of radiation on (1) critical flicker frequency (vision) (2) conditioned emotional responses (emotional behavior) and (3) tone discrimination (auditory). Using many of these same methods additional studies have been conducted on rats which had been exposed to ionizing radiations during pre-natal development.

Progress: During the progress of this investigation, facilities for the simultaneous housing and training of ten animals have been constructed at the Behavioral Radiology Laboratory, Forest Glen Section, WRAMC. Equipment includes automatic timers, counters, programming devices, recorders, and a punch tape recording system compatible with existing computer facilities, all of which are necessary for the complex experimental analysis of behavior. The first phase of the project has been completed and yielded data on the time dose relationships that were necessary for the design and on the second phase. In addition, a perfect rank correlation between the amount of pre-exposure rest from the stressful avoidance conditions and survival times was found. A formal study to further investigate this phenomenon has recently been completed. The results of this study indicate that there is a complex relationship between amount of rest, the duration of exposure to the avoidance schedule and survival times. Using a dose of X-rays of 10,000 r animals which had been stressed and rested or simply stressed lived longer than non stressed control animals.

The second phase, in accordance with the original proposal, has been initiated and base-line studies and equipment testing conducted. In addition arrangements have been made with AFFRI for a comparative study of the effects of mixed radiations and X-rays.

This study will also afford a comparison of behavioral and biological effects between pulsed exposures and exposures at much slower rates.

The findings of the rat studies suggest that sub-lethal doses of X-ray delivered to the whole body (1) do not alter the critical flicker frequency, (2) slightly decrease pure tone discrimination performance and (3) markedly alter the emotional properties of a painful electric shock as tested by the conditioned emotional response paradigm.

Summary and Conclusions: Methods have been developed for the assessment of sensory capacities and motor coordination in primates. Also, the data acquisition and reduction problems are being handled by high speed paper punch tapes and existing computer facilities. These methods will provide highly reliable data from relatively few animals. Studies on rats suggest that at sub-lethal doses, vision, as measured by critical flicker fusion, is less radio sensitive than the auditory sense as measured by tone discrimination. The alteration in the emotional behavior of rats following an exposure to sub-lethal X-rays suggest a very radio sensitive biological mechanism.

ARMY RESEARCH TASK REPORT		REPORTS CONTROL SYMBOL CSCRD-4(R2)
ACCESSION NUMBER 37355	PROJECT, TASK, OR SUBTASK NO. RD 41-61 c 03.037	
1. REQUESTING AGENCY US Army Combat Development Command; Nuclear Group Fort Bliss, Texas	2. FUNDING AGENCY Defense Atomic Support Agency Department of Defense Washington, D. C., 20315	
3. CONTRACTING AGENCY NA	4. CONTRACTOR AND/OR GOV'T LABORATORY A Walter Reed Army Inst of Rsch Walter Reed Army Medical Center Washington, D. C., 20012 723-1000, Ext 3552	
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Davidson, David E., Capt, VC, Dept of Medicinal Chemistry, Special Activity, WRAIR, WRAMC, Washington, D. C., 20012 576-3568 or Interdepartmental Code 198, Ext 3568, See Continuation Sheet		
6. TITLE OF: PROJECT <input checked="" type="checkbox"/> (U) TASK <input checked="" type="checkbox"/> SUBTASK <input checked="" type="checkbox"/> Life span studies in irradiated dogs. (U)		
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964		
8. RESUME' (U) Long term observation and laboratory investigation of dogs surviving massive acute or fractionated whole body irradiation was continued.		
9. KEY WORDS Life span, gamma, irradiation, nuclear weapons effects, radiobiology, blood urea nitrogen		
10. SUPPORTING PROJECTS NA		
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet	12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet	
<div style="display: flex; justify-content: space-between;"> DA FORM 1309R 1 June 63 PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of _____ </div>		

ACCESSION NO.

37355

ARMY RESEARCH TASK REPORT

13. PROJECT, TASK OR
SUBTASK NUMBER

11	22	23 24	25 26	27 28	29
R D 4 1 - 6 1 C 0 3	0	0	3 7		

14. DATE OF REPORT (30-33)

30	33	34
0 6 6 4	4	

15. SECURITY OF WORK (34)

16. TYPE OF REPORT

35	36	47 48 49 50 51	52	55
2			0 6 6 3	

17. SCIENTIFIC FIELD

 a. Topical Classific. (56-61)
 b. Functional Class (62-64)

56	61	62	64
0 1 0 1 0 0			

18. OSD CLASSIFICATION
(65-66)

19. R&D CATEGORY (67)

65 66	67
A R	1

20. CONTRACT NUMBER

11 12	13 14	15 17	18	21	22	26	27
D A							

21. GRANT NUMBER

28 29	30	33	34 35	36	38	39 40	41	45	46
D A							G		

22. ESTIMATED COMPLET.
DATES

47	51	52	56	57	61	62	66	67	71
1 0 6 6 4	2			3		4		5	

23. PRIORITY (11-14)

24. PROGRAM ELEMENT
(15-26)

11	14	15	26

25. CMR&D CODES

27	29	30	32	33	35

26. CDOG REFERENCE

 a. Paragraph No. (36-44)
 b. Functional Group (45)

36	39	40	41 42	43 44	45
					0

27. FUNDING

 a. Est. Total Cost (11-15)
 b. % Spent Intern. (16-18)
 " " Extern. (19-21)
 c. Total Obligation (22-26)
 d. Progrmd. Cur. FY (27-33)
 e. " " " +1 (34-40)
 f. " " " +2 (41-47)
 g. " " " +3 (48-54)
 h. " " " +4 (55-61)
 i. " " " +5 (62-68)
 j. " " " +6 (69-75)
 k. Total Man Years of
 Effort (76-78)

11	15	16 18	19 21	22	26
	1 0	1 0 0	2		1 7 1
27 28	29	33	34 35	36	40
6 4		1 0			
41 42	43	47	48 49	50	54
55 56	57	61	62 63	64	68
69 70	71	75	76	78	
				0	

ACCESSION NUMBER

37355

ARMY RESEARCH TASK REPORT
Continuation Sheet

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

(A) Woodward, Kent T., Lt Col, MC, Director, Div of
Nucl Med, WRAIR, WRAMC, Washington, D. C., 20012
576-2211 or Interdepartmental Code 198, Ext 2211

49

REPORTS. Annual Progress Report, Walter Reed Army Institute of
Research, 1 July 1963 - 30 June 1964.

DA FORM 1309R
1 June 63

PREVIOUS EDITIONS ARE OBSOLETE

Page of _____

ANNUAL PROGRESS REPORT

Project No. RD 44-61

Title: Biomedical (NWER)

Task No. 03.037

Title: Life span studies in

Subtask No. 03.037

Title: Life span studies in
irradiated dogs

Description: Survivors of massive acute or fractionated whole-body irradiations were maintained at the Walter Reed Army Institute of Research. University of Rochester personnel were assisted by Institute personnel in investigations of late effects.

Progress: Thirty-four of a group of irradiated dogs originally numbering fifty are being observed and evaluated. These dogs are survivors of whole-body radiation experiments conducted at the University of Rochester during 1955-57 using x-rays or cobalt-60 gamma rays in single or fractionated doses. The dogs have variously received cumulative doses of 200 r to 1400 r. Periodic clinical and hematologic examinations have been performed during the past year. Selected tests of organ function have been initiated.

Hemograms of the colony are in general, normal. Blood urea nitrogen was slightly elevated in only a few dogs.

The general clinical condition of this colony is declining gradually due to advancing age. Skin and dental problems are increasing. Twelve deaths have occurred in the past year - 3 due to pyometra, one chronic nephritis, one acute leptospiral nephritis, one pneumonia, one fatal fight injury, one renal and cystic urolithiasis, one gastrointestinal malignancy, and one of undetermined etiology.

Several additional benign neoplasms were observed, some of which were surgically removed - two mammary tumors, one tumor of the hard palate, two papillomas of the external ear and 5 perianal adenomas (one in a female).

Observation of the remaining dogs is continuing for their life span.

Summary and Conclusions: Clinical observation and laboratory investigation of dogs surviving large doses of gamma irradiation were continued.

ARMY RESEARCH TASK REPORT		REPORTS CONTROL SYMBOL CSCRD-4(R2)
ACCESSION NUMBER 37356		PROJECT, TASK, OR SUBTASK NO. RD 41-61 c 03.038
1. REQUESTING AGENCY US Army Combat Development Command Nuclear Group Fort Bliss, Texas	2. FUNDING AGENCY Defense Atomic Support Agency Department of Defense Washington, D. C., 20315	
3. CONTRACTING AGENCY NA	4. CONTRACTOR AND/OR GOV'T LABORATORY A Walter Reed Army Inst of Rsch Walter Reed Army Medical Center Washington, D. C., 20012 723-1000, Ext 3552	
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Woodward, Kent T., Lt Col, MC, Dir, Div of Nuclear Medicine WRAIR, WRAMC, Washington, D. C., 20012 576-2211 or Interdepartmental Code 198, Ext 2211 See Continuation Sheet		
6. TITLE OF: PROJECT <input type="checkbox"/> TASK <input type="checkbox"/> SUBTASK <input checked="" type="checkbox"/> Metabolism of fission products from fallout (U)		
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964		
8. RESUME (U) The objective of this subtask is to determine the effects of external radiation, wounds, illnesses, etc., on the metabolism of internal emitters, the goal being to develop methods by which the introduction, uptake, and excretion of fission products can be altered to the benefit of the individual. Without adequate prophylactic and clinical controls, the pathological effects of radiation from internally deposited radionuclides could, even in a nuclear war, ultimately surpass in importance the hazards from external radiation. In this study, information is being obtained by surveillance of workers with accidental or occupationally acquired body burdens as well as from data obtained in studies of laboratory animals and human volunteers after specific radio-nuclide administration. Data is also being accumulated about the exchange of fission and activation products between man and his environment. These studies, which depend on the human whole-body and low level radioactivity counters at the Walter Reed Army Institute of Research and on collaboration and coordination with related research and clinical efforts at WRAIR and at Walter Reed General Hospital, include acute metabolism of various radionuclides in the rat; long term whole-body turnover of trivalent chromium in rats; dogs and man; whole-body turnover of manganese and chromium in irradiated rats; surveillance of 250 occupationally exposed persons.		
9. KEY WORDS Fallout, fission products, fission product metabolism, health physics, industrial hygiene, nuclear weapons effects, whole-body counting.		
10. SUPPORTING PROJECTS Not Applicable		
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet	12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet	
<div style="display: flex; justify-content: space-between;"> <div>DA FORM 1309R 1 June 63</div> <div>PREVIOUS EDITIONS ARE OBSOLETE</div> <div>PAGE 1 of _____</div> </div>		

ACCESSION NO.		ARMY RESEARCH TASK REPORT	
37356			
13. PROJECT, TASK OR SUBTASK NUMBER	11 22 23 24 25 26 27 28 29 R D 4 1 - 6 1 C 0 3 . 0 3 8		
14. DATE OF REPORT (30-33)	30 33 34 0 6 6 4 4		
15. SECURITY OF WORK (34)			
16. TYPE OF REPORT	35 36 47 48 49 50 51 52 55 2		
17. SCIENTIFIC FIELD a. Topical Classific. (56-61) b. Functional Class (62-64)	56 61 62 64 0 1 0 7 0 0		
18. OSD CLASSIFICATION (65-66)	65 66 67 A R 1		
19. R&D CATEGORY (67)			
20. CONTRACT NUMBER	11 12 13 14 15 17 18 21 22 26 27 D A		
21. GRANT NUMBER	28 29 30 33 34 35 36 38 39 40 41 45 46 D A G		
22. ESTIMATED COMPLET. DATES	47 51 52 56 57 61 62 56 67 71 1 0 6 6 4 2 3 4 5		
23. PRIORITY (11-14)	11 14 15 26		
24. PROGRAM ELEMENT (15-26)			
25. CMR&D CODES	27 29 30 32 33 35		
26. CDOG REFERENCE a. Paragraph No. (36-44) b. Functional Group (45)	36 39 40 41 42 43 44 45 0		
27. FUNDING a. Est. Total Cost (11-15) b. % Spent Intern. (16-18) " " Extern. (19-21) c. Total Obligation (22-26) d. Progrmd. Cur. FY (27-33) e. " " " +1 (34-40) f. " " " +2 (41-47) g. " " " +3 (48-54) h. " " " +4 (55-61) i. " " " +5 (62-68) j. " " " +6 (69-75) k. Total Man Years of Effort (76-78)	11 15 16 18 19 21 22 26 . . . 5 6 1 0 0 2 . . . 9 2 27 28 29 33 34 35 36 40 6 4 . . . 3 0 41 42 43 47 48 49 50 54 55 56 57 61 62 63 64 68 69 70 71 75 76 78		

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

- (A) Blanton, T. R., Sp5, Dept of Isotope Metabolism,
Div of Nucl Med, WRAIR, WRAMC, Washington, D. C., 20012
576-3305 or Interdepartmental Code 198, Ext 3305 49
- (A) George, James, Capt, MC, Dept of Hematology, Div of
Medicine, WRAIR, WRAMC, Washington, D. C., 20012
576-3060 or Interdepartmental Code 198, Ext 3060 49
- (A) Leitnaker, F. C., Maj, MC, Dept of Isotope Metabolism,
Div of Nucl Med, WRAIR, WRAMC, Washington, D. C., 20012
576-3023 or Interdepartmental Code 198, Ext 3023 49
- (A) Pollack, Simeon, Capt, MC, Dept of Hematology, Div of
Medicine, WRAIR, WRAMC, Washington, D. C., 20012
576-3060 or Interdepartmental Code 198, Ext 3060 49
- (A) Reba, Richard C., Maj, MC, Dept of Isotope Metabolism,
Div of Nucl Med, WRAIR, WRAMC, Washington, D. C., 20012
576-3305 or Interdepartmental Code 198, Ext 3305 49
- (A) Uhrig, H. T., Maj, MC, Dept of Isotope Metabolism,
Div of Nucl Med, WRAIR, WRAMC, Washington, D. C., 20012
576-3305 or Interdepartmental Code 198, Ext 3305 49

REPORTS. Annual Progress Report, Walter Reed Army Institute
of Research, 1 July 1963 - 30 June 1964.

ANNUAL PROGRESS REPORT

Project No. DASA RD 41-61

Title: Biomedical (NWER)

Subtask No. 03.038

Title: Metabolism of fission products
from fallout

Description:

1. Gastrointestinal absorption and clearance, and liver uptake of radionuclides in rats was measured.
2. Chromium and manganese turnover in normal humans and laboratory animals.
3. Chromium and manganese turnover in irradiated laboratory animals.
4. Military and industrial hygiene surveillance of radionuclide body burdens in human subjects from occupationally exposed groups and from the general population.

Progress:

1. Using a technique developed in the Department of Hematology, Division of Medicine, WRAIR, the six-hour gastrointestinal absorption of orally administered radionuclides of the following elements was studied: calcium, cesium, chromium, cobalt, copper, iron, manganese, magnesium, mercury and zinc. In some cases, the six-hour gastrointestinal clearance after intravenous injection was also studied. Liver uptake was also determined in selected instances. Dose-response was investigated.

After administration of the radionuclide (oral or intravenous) the six-hour total-body retention was determined. A partial summary of the data collected is listed in the accompanying chart.

Liver uptake using manganese at three dose levels after both oral and IV administration was also studied. Liver blood pool was estimated following injection of Chromium-51 labeled albumin.

Table 1: Six-Hour Total-Body Retention (% Dose \pm 1 S. D.)

	<u>Per Os</u>		<u>I. V.</u>	
	<u>"Carrier-Free"</u> <u>Isotope</u>	<u>with 5 uM</u> <u>"Carrier"</u>	<u>"Carrier-Free"</u> <u>Isotope</u>	<u>with 5 uM</u> <u>"Carrier"</u>
Calcium	64.0 \pm 21.7	35.0 \pm 7.5	93.6 \pm 3.7	90.2 \pm 2.8
Cesium		77.7 \pm 2.6		
Chromium				
Cobalt		18.4 \pm 6.7		
Copper	9.6 \pm 2.9	5.7 \pm 4.7	83.0 \pm 4.6	
Iron		11.5 \pm 4.8		
Manganese	2.9 \pm 2.9	3.0 \pm 2.0	74.1 \pm 11.9	28.2 \pm 7.4
Magnesium		42.0 \pm 5.5		
Mercury		11.2 \pm 7.0		
Zinc	16.9 \pm 5.9	14.5 \pm 6.7		

2. Total-body counting of dog, man, and rat after intravenous Chromium-51 administration was followed for three months. The effect of the route of administration was evaluated. Excretion studies in selected animals were carried out for up to 21 days. The total-body retention was evaluated by simple compartmental analysis. A summary of these results is listed in the following chart.

Table 2:

	<u>Effective $t_{1/2}$ (days)</u>			<u>Compartment Size (% dose)</u>		
	<u>Dog</u>	<u>Man</u>	<u>Rat</u>	<u>Dog</u>	<u>Man</u>	<u>Rat</u>
C	20	21	20	20	10	26
B	5	6	5	10	6	34
A	0.6	0.5	0.6	70	84	40

3. Two months following LD₅₀ whole-body X-irradiation, Wistar rat survivors were injected with radioactive manganese and chromium and whole-body counting was performed. The radiomanganese turnover was clearly more rapid over the 30-day observation period than in a control group. Chromium turnover was also more rapid than the controls but only for the first

three-four days. From the fifteenth through the termination of the study (30 days) the total body clearance was distinctly delayed.

4. Surveillance by whole-body counting is maintained of military personnel having occupational contact with gamma-emitting nuclides at nearby installations. Since whole-body counting may detect gamma activity induced by neutron fluxes, base line counts are taken of personnel working around reactors. All personnel on post are counted as a means of sampling the general population, world-wide, for delayed fallout. Subjects suspected of having elevated burdens of gamma-emitters, by history or after counting in HUMCO (the liquid scintillator whole-body counter), are subjected to gamma-ray spectrometry.

Gamma radioactivity was surveyed in occupationally-exposed subjects from the following organizations:

National Bureau of Standards Personnel. During FY64, 123 subjects who handle gamma-emitters were counted in HUMCO. Ten of these were also examined with the 9"(D) x 4" NaI Crystal Whole-Body Counter.

WRAIR Personnel. Ninety-eight reactor personnel and other radiation workers exposed to fission products and other gamma-emitters have been counted this FY.

Diamond Ordnance Fuze Laboratory. Thirteen reactor section personnel of this laboratory were examined.

Edgewood Arsenal. Five subjects concerned with disposal of radioactive wastes and/or radioisotopes in a laboratory were checked.

None of the above groups had detectable levels of contamination.

Budd Company, Philadelphia, Pennsylvania. Eleven employees were examined in HUMCO. Body burdens of Cobalt-60 were easily detected in all and verified in three by crystal spectrometry and exposure history. The largest burden was estimated to be less than 0.1 the maximal permissible body burden.

A total of 358 subjects exposed to no gamma-emitters except world-wide fallout from weapon testing were counted in HUMCO. There has been a steady increase in Cs-137 body burdens since resumption of this study in July 1963. However, body burdens do not exceed 1-100th of that allowable for the general population.

Summary and Conclusions:

1. Selected Radionuclide Metabolism in Rats. Data on the acute metabolism of ten selected radionuclides was accumulated. This work is being evaluated and will be studied further.
2. Chromium Metabolism in Normal Rats. The effective disappearance time in the three species studied appeared to be the same; only the compartmental size varied. The effective half-time measured in these studies is at **variance** with the reported figures listed in the current ICRP report.
3. Clearance Patterns in Irradiated Animals. There was a significant difference in the pattern of clearance of chromium and manganese in the irradiated survivors as compared to normal controls.
4. Human Radionuclide Burdens. Eleven of 250 radiation workers counted had detectable increases of body burden. A total of 358 members from the general population were monitored for Cesium-137 burdens, which, although rising, are two orders of magnitude below RPC values.

ARMY RESEARCH TASK REPORT		REPORTS CONTROL SYMBOL CSCRD-6(R2)
ACCESSION NUMBER 37357	PROJECT, TASK, OR SUBTASK NO. RD 41-61 c 03.074	
1. REQUESTING AGENCY US Army Combat Development Command Nuclear Group, Fort Bliss, Texas	2. FUNDING AGENCY Defense Atomic Support Agency Department of Defense Washington, D. C., 20315	
3. CONTRACTING AGENCY NA	4. CONTRACTOR AND/OR GOV'T LABORATORY A Walter Reed Army Inst of Rsch Walter Reed Army Medical Center Washington, D. C., 20012 723-1000, Ext 3552	
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Woodward, Kent T., Lt Col, MC, Dir, Div of Nuclear Medicine WRAIR, WRAMC, Washington, D. C., 20012 576-2211 or Interdepartmental Code 198, Ext 2211 See Continuation Sheet		
6. TITLE OF: PROJECT <input type="checkbox"/> TASK <input type="checkbox"/> Biological effects of total and partial body irradiation SUBTASK <input checked="" type="checkbox"/> at cellular, organ, and total organism level (U)		
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964		
8. RESUME (U) The object of this subtask is the understanding of radiation injury in man. Investigations of physiological, pathological, and clinical manifestations of radiation effects on cells, organs, and total organisms are aimed at development of protective measures and specific countermeasures. Areas are (1) effectiveness of shielding in a combat environment, (2) effects of exposure to sublethal doses of neutron, gamma, and mixed radiations on combat efficiency, (3) roles of metabolism, environment, and infectious processes in development of late effects of radiation. (4) discovery of biomedical parameters applicable to prediction of nuclear warfare casualties and (5) determination of field medical treatment requirements in nuclear war. Current efforts under this subtask are (1) effects of pooled allogeneic bone marrow on the course of secondary radiation syndrome, (2) late effects of total body irradiation, (3) response of irradiated dogs to vaccination with attenuated viruses, (4) protection by sphere-grid shielding and the role of healthy tissue surrounding irradiated areas, (5) relationship between radiosensitivity and cell proliferation of L-strain mouse fibroblasts <u>in vitro</u> , (6) influence of growth phase and macromolecular syntheses on radiation killing and recovery of <u>E. coli</u> , (7) detection and measurement of transient free radicals in irradiated nucleic acids and their components, <u>in vitro</u> .		
9. KEY WORDS Bacteria, bone marrow, free radicals, growth, ionizing radiation, late effects, mammals, nuclear weapons effects, nucleic acids, partial-body, proteins, radiation sensitivity, recovery, sphere-grid, total-body, vaccination.		
10. SUPPORTING PROJECTS NA		
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet	12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet	
<div style="display: flex; justify-content: space-between;"> DA FORM 1309R 1 June 63 PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of ____ </div>		

ACCESSION NO. 37357		ARMY RESEARCH TASK REPORT	
13. PROJECT, TASK OR SUBTASK NUMBER	11	22	23 24 25 26 27 28 29
	R D 4 1 - 6 1 c 0 3	. 0	7 4
14. DATE OF REPORT (30-33)	30	33	34
15. SECURITY OF WORK (34)	0 6 6 4		4
16. TYPE OF REPORT	35	36	47 48 49 50 51 52 55
	3		0 6 6 3
17. SCIENTIFIC FIELD a. Topical Classific. (56-61) b. Functional Class (62-64)	56	61	62 64
	0 1 0 2 0 0		
18. OSD CLASSIFICATION (65-66)	65 66	67	
19. R&D CATEGORY (67)	A R	1	
20. CONTRACT NUMBER	11 12	13 14	15 17 18 21 22 26 27
	D A		
21. GRANT NUMBER	28 29	30	33 34 35 36 38 39 40 41 45 46
	D A		
22. ESTIMATED COMPLET. DATES	47	51	52 56 57 61 62 66 67 71
	1 0 6 7 0	2	3 4 5
23. PRIORITY (11-14)	11	14	15 26
24. PROGRAM ELEMENT (15-26)			
25. CMR&D CODES	27 29	30 32	33 35
26. CDOG REFERENCE a. Paragraph No. (36-44) b. Functional Group (45)	36	39	40 41 42 43 44 45
			0
27. FUNDING a. Est. Total Cost (11-15) b. % Spent Intern. (16-18) " " Extern. (19-21) c. Total Obligation (22-26) d. Progrmd. Cur. FY (27-33) e. " " " +1 (34-40) f. " " " +2 (41-47) g. " " " +3 (48-54) h. " " " +4 (55-61) i. " " " +5 (62-68) j. " " " +6 (69-75) k. Total Man Years of Effort (76-78)	11	15	16 18 19 21 22 26
		6 9	1 0 0 2 2 3 0
	27 28	29	33 34 35 36 40
	6 4		6 5 5
	41 42	43	47 48 49 50 54
	6 6		6 7 9 5
	55 56	57	61 62 63 64 68
	6 8		6 9 9 5
	69 70	71	75 76 78
	3 0		9

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

- (A) Berman, A. R., B.S., Dept of Isotope Metabolism
Div of Nuc Med, WRAIR, WRAMC, Washington, D. C., 20012
576-3020 or Interdepartmental Code 198, Ext 3020 49
- (A) Blom, J., M.D., Hematology Clinic
WRGH, WRAMC, Washington, D. C., 20012
576-3486 or Interdepartmental Code 198, Ext 3486 49
- (A) Davidson, D. E., Capt, VC, Dept of Medicinal Chemistry
WRAIR, WRAMC, Washington, D. C., 20012
576-2280 or Interdepartmental Code 198, Ext 2280 49
- (A) Davis, M. H., B.S., Dept of Radiation Biology
Div of Nuc Med, WRAIR, WRAMC, Washington, D. C., 20012
576-3019 or Interdepartmental Code 198, Ext 3019 49
- (A) Ginsberg, D. M., Maj, MSC, Dept of Biophysics
Div of Nuc Med, WRAIR, WRAMC, Washington, D. C., 20012
576-3017 or Interdepartmental Code 198, Ext 3017 49
- (A) Glinos, A. D., M.D., Dept of Cellular Physiology
Div of Basic Surg Rsch, WRAIR, WRAMC, Wash D. C., 20012
576-5284 or Interdepartmental Code 198, Ext 5284 49
- (A) Knospe, W. H., Maj, MC, Dept of Radiation Biology
Div of Nuc Med, WRAIR, WRAMC, Washington, D. C., 20012
576-3023 or Interdepartmental Code 198, Ext 3023 49
- (A) Krebs, A. T., Ph.D., Dept of Biophysics
Div of Nuc Med, WRAIR, WRAMC, Washington, D. C., 20012
576-3019 or Interdepartmental Code 198, Ext 3019 49
- (A) Lown, J. W., Ph.D., Dept of Biophysics
Div of Nuc Med, WRAIR, WRAMC, Washington, D. C., 20012
576-3782 or Interdepartmental Code 198, Ext 3782 49
- (A) McConnell, S. J., Maj, VC, Dept of Vet Microbiology
Div of Vet Med, WRAIR, WRAMC, Washington, D. C., 20012
576-3520 or Interdepartmental Code 198, Ext 3520 49
- (A) North, H. H., B.S., Dept of Cellular Physiology
Div of Basic Surg Rsch, WRAIR, WRAMC, Wash D. C., 20012
576-5307 or Interdepartmental Code 198, Ext 5307 49
- (A) Spertzel, R. O., Capt, VC, Dept of Vet Microbiology
Div of Vet Med, WRAIR, WRAMC, Washington, D. C., 20012
576-3520 or Interdepartmental Code 198, Ext 3520 49
- (A) Wampler, S. N., Capt, VC, Dept of Food Radionuclides
Div of Vet Med, WRAIR, WRAMC, Washington, D. C., 20012
576-3646 or Interdepartmental Code 198, Ext 3646 49

REPORTS. Annual Progress Report, Walter Reed Army Institute of Research, 1 July 1963 - 30 June 1964.

Ginsberg, D. M.: The Influence of Metabolic State and Growth Phase on Ultraviolet Killing of Escherichia coli Strain 15T⁻A⁻U⁻. Ph.D. Thesis, The University of Tennessee, Knoxville. 109 p. 1963.

Ginsberg, D. M., and Jagger J.: Radiation Sensitivity and Growth Characteristics of an Arginine- and Uracil-Deprived Culture of Escherichia coli Strain 15T⁻A⁻U⁻, submitted to J. Gen. Microbiol., 1964.

Ginsberg, D. M., and Jagger, J.: Evidence that Initial Ultraviolet Lethal Damage in Bacteria is Independent of Growth Phase, submitted to Biophysical J., 1964.

Glinos, A. D., and North, H.: Cellular Growth and Tissue Radio-sensitivity: Cell Studies in vitro and General Concepts. Trans. N. Y. Acad. Sci., 26:145-158, 1963.

Knospe, W. H., Blom, J., and Davis, M. H.: Administration of Pooled Allogenic Bone Marrow to Lethally Irradiated Rats. Fed. Proc., (in press) 1964.

ANNUAL PROGRESS REPORT

Project No. DASA RD 41-61

Title: Biomedical (NWER)

Subtask No. 03.074

Title: Biological effects of total and partial body irradiation at cellular, organ, and total organism levels

Description:

1. Effectiveness of pooled bone marrow from single and multiple donors in reducing the complications (graft-host immunologic reactions) of bone marrow allografts after total-body lethal irradiation of mammals.
2. Long-term post-irradiation clinical examinations, routine hematological tests, and systematic organ function studies to observe the possible development of late effects in survivors of total-body exposures to ionizing radiation.
3. Evaluation of pre- and post-irradiation vaccination to determine the effects of total body irradiation of the host on (a) the susceptibility and immunologic recall mechanisms in the host and (b) the virulence and immunologic properties of attenuated live-virus vaccines.
4. Irradiation of small mammals through sphere-grids (partial-body irradiation) to investigate the effects of unirradiated, healthy tissue immediately adjacent to heavily irradiated areas on ultimate radiation pathology.
5. Relationship between radiation sensitivity of a mammalian cell population in vitro and the growth and proliferation of individual cells in the population.
6. Metabolic parameters influencing the capacity of bacteria to recover from radiation lethal damage.
7. Development and exploitation of techniques using magnetic resonance spectroscopy to study the nature, site, and modification of initial radiation lesions.

Progress:

1. Bone Marrow Studies. Studies were designed to evaluate the effect of pooled allogeneic bone marrow upon the course of the secondary syndrome. Sixty female Wistar rats were randomized into four groups of 15 animals each. Group A received no treatment. Groups B, C and D received 1000r total body irradiation from a Co-60 source. Group B received no marrow.

Each animal of Group C received ($41-102 \times 10^6$) allogeneic nucleated cells I.P. from a single donor five hours post-irradiation. The 15 animals of Group D were divided into three equal subgroups. Each of these five animals received pooled allogeneic marrow I.P. from five donors ($30-60 \times 10^6$ cells). All animals in this experiment were randomly bred to preclude inbreeding.

No animals in Group A died. All animals in Group B died by day 15. At 30 days, 14 of 15 animals in Group C and nine of 15 animals in Group D survived. At 60 days ten animals in Group C and eight animals in Group D survived. Average weights of Groups C and D have been virtually identical throughout the post-irradiation period of nine months to the present time. Average weights of C and D have lagged significantly behind Group A throughout the period of observation. None of the animals of Group C and Group D have demonstrated other signs of secondary disease.

These results raised the question of a weak or absent histocompatibility barrier in the utilized strain in spite of random breeding. A second experiment was designed utilizing pooled allogeneic bone marrow derived from individuals of five different strains. Single donor marrow was obtained from Sprague-Dawley rats and pooled marrow was obtained from Sprague-Dawley, Fisher, NIH Black, Long-Evans and Sherman rat strains. The experimental design was similar to the first experiment except that the number of animals in each group was increased to 20. Marrow was injected into tail veins of Wistar females given 825r total body X-irradiation (300 KVP) five hours previously.

In the second experiment, all animals in Group A survived and all animals in Group B died within 16 days. Fourteen animals of Group C and 13 animals of Group D survived at seven weeks post-irradiation. So far these two groups do not differ significantly in their average weights but remain below the controls.

2. Canine Studies. Thirty-seven purebred beagle dogs surviving whole body 1-MEV X-irradiation of 400 to 750r at 1r/minute to 10r/minute during the winter of 1961-1962 were being observed for the possible development of late effects. Frequent clinical examinations, routine hematologic studies, and systematic organ function studies have been performed. The dogs continue to be in generally good clinical condition. Greying of the hair in the irradiated groups is apparent. Obesity has been observed in several of the irradiated dogs, but not in the unirradiated control group. Anemia due to hookworm infestation or pregnancy has been encountered and corrected. Hysterectomies have been performed on all females, and renal biopsies obtained. Thyroid studies, including Iodine-131 uptake curves were performed at one year post-irradiation. No abnormalities in thyroid function were observed. Total body water and total body potassium determination by tritium tracer and whole-body counting techniques respectively have revealed no changes attributable to

irradiation. Hematologic function using Iron-59 and Chromium-51 tracers has been studied at 13 to 15 months post-irradiation. Total blood volume, red cell mass, plasma iron clearance, and red cell lifespan have been measured. No significant differences between the irradiated and control dogs were observed. Peripheral hematologic determinations, however, continue to show gradual improvement in the irradiated groups. Blood urea nitrogen determinations in October 1963 were all within normal limits.

3. Vaccine Studies. The preceding annual report contains descriptions of the experimental procedures and the results of the bulk of the experimental work in this study. A final report is being prepared. Results of experiments not previously reported show that rabies neutralizing antibodies developed in all vaccinated dogs following vaccination and in all susceptible dogs dying of rabies virus infection following challenge with a virulent rabies virus. Two non-vaccinated dogs (one irradiated and one non-irradiated) with no neutralizing antibodies at time of challenge, survived the virus challenge. The irradiated dog developed high level neutralizing antibodies 10 days post challenge, whereas the non-irradiated animal developed no neutralizing antibody.

4. Grid Shielding. The beneficial effects of sieve irradiations in the treatment of deep seated tumors are well established, even if the mechanism(s) is not yet known. A similar situation exists in grid protection studies, where animals shielded by grids of proper dimensions (open-to-closed area, hole diameter) can be exposed to radiation fields many factors higher in dose than the fields which would produce the same effect without properly dimensioned grids.

The theory presently most favored to explain the "grid-phenomena" deals with the circulatory and vasculatory processes going on in and at the interface area between irradiated tissue cylinders (positive grids) and unirradiated tissue masses. Arguments so far are based primarily on histopathological findings. Practically nothing is known about these processes in fresh living tissue. This problem is being studied using histochemical techniques for detection and demonstration of the functional changes, fluorescence microscopy, and autoradiography.

In experiments with positive grids (40-50% open-closed-area; 3.6 mm thick lead shields; 4.4 mm hole sizes; 1.9 mm hole sizes), exposure of small mammals to radiation fields of up to 6000r have proved the advantages of small hole sizes. Preliminary data indicate that animals shielded by properly constructed sphere grids and exposed to radiation fields of about 6000r die in the same time and in the same position (death-sequence) as animals exposed, unshielded, to 1000r.

Grid-irradiated C-57 black mice show greying of hair in grid-correlated patterns on both proximal and distal (with respect to orientation of the animals to the radiation source) surfaces. Grid patterns

were also observed on the surface of fresh spleen and fresh kidneys of mice following grid-irradiation without any chemical pre-treatment or histological preparation. Experiments designed to study the events leading to these "tissue-patterns" at different times after exposure to high-dose-radiation fields are going on. Using a shield constructed of shotgun pellets, sphere-grid pattern epilation was also observed in irradiated ICR mice.

Tetrazolium compounds are being used to measure the dynamic, functional changes in vascular and circulatory processes. In addition, acridine-orange-stained preparations of fresh tissue will be examined for histological changes. Other changes may be demonstrated by autoradiography experiments.

5. Cell Culture Studies. Studies on the sensitivity to ionizing radiation of logarithmic and stationary phase mammalian cells in vitro have continued. It was observed that the radiosensitivity of clone-forming ability is the same in stationary and logarithmic mammalian cell cultures, suggesting that the injury to the genetic apparatus is identical in both cultures. Irradiated cells in which clone-formation is inhibited can, nevertheless, undergo a limited number of post-irradiation divisions. The number of divisions completed by these cells is inversely proportional to the radiation dose. The linearity of this dose-effect relationship has been shown for both logarithmic and stationary cells. However, for the same dose, logarithmic origin cells completed a significantly higher number of divisions than stationary origin cells.

6. E. coli Studies. Previously reported studies using E. coli strain 15TAU (requires thymine, arginine, and uracil for normal growth) indicate that the kinds and amounts of initial radiation lethal damage are not significantly different in logarithmic- and stationary-phase cells, even though stationary-phase cells show a much higher survival after any given dose of radiation. It was concluded that the radiation sensitivity of bacterial populations irradiated in different growth phases is related to the ability of the cells to recover from the damage, rather than to differences in primary lethal lesions.

The observation, not previously reported, that the delays in resumption of DNA synthesis and cell division of control cells plated on routine assay medium (nutrient agar) were roughly parallel to the degree of radiation resistance observed for the respective growth phases, is consistent with the earlier conclusion.

These findings and conclusions led to experiments currently in progress to characterize certain metabolic response in E. coli 15TAU populations which show different radiation sensitivities.

result of arginine- and uracil-starvation-induced, non-specific incapacities to make proteins required for synthesis of DNA and other macromolecules) Studies are currently underway to determine whether "orientation" of DNA synthesis occurs under the various conditions of growth known to modify radiation survival. These studies include (1) analysis of growth kinetics and (2) use of pulsed radioactivity labeling and buoyant density analysis in a gradient centrifuge to observe the sequential replication of DNA

Accumulating data from bacterial-irradiation experiments have revealed an apparent mathematical relationship between certain biological parameters (e.g., growth lag times, and capacities for initiating or resuming DNA synthesis) and observed survival. These relationships suggest that capacity to "repair" initial radiation damage can be measured in control populations. Theoretical and experimental evaluation of this concept is underway to determine whether these biological parameters, when measured accurately, might be used to predict the radiation response of a bacterial population.

7. Decomposition of Nucleosides. Since it appears likely that the most damaging lesion is associated with molecules directly connected with genetic processes, the work in progress concerns the radiation chemistry of the nucleic acids and their components in vitro. Methods for identifying the lesion sites in DNA isolated from irradiated cells are being studied. In addition, a systematic study of the radiation damage to nucleosides, nucleotides and, ultimately, nucleic acids, in the presence of a variety of protectants, is underway.

An approximately linear relationship exists between percent decomposition and radiation dose for 10^{-3} M aqueous solutions of thymine, adenine and adenosine. The response is similar in kind in the presence of equimolar amounts of modifying agents, but the extent of decomposition is reduced drastically with cystamine and cysteamine, with a much greater degree of protection afforded by the aminothiols. A lesser but substantial degree of modification is afforded by specific binding of the bases of mercuric ion. Analysis of the decomposition of adenosine is being carried out by separation of free adenine as the silver complex, followed by conversion to the picrate, and, finally, titration of the base-picric acid complex.

A preliminary study, using nuclear magnetic resonance spectroscopy, was designed to elucidate the mode of bonding of mercury to nucleosides. However, the utility of this method will depend on being able to isolate DNA in an intact form from irradiated materials. The possibility exists of identifying intermediate free radicals formed by the action of primary radicals on the purine, pyrimidine, and ribofuranoside moieties. A flow apparatus using the radiometric system $\text{TiCl}_3/\text{H}_2\text{SO}_4/\text{H}_2\text{O}_4$ has been developed.

Measurements have been made of the rate of DNA synthesis and the amount of DNA per cell in different growth phases. Comparing logarithmic- and stationary-phase cells, no significant difference in amount of DNA per cell can be detected with certainty. The data show that, if any difference does exist, there is slightly less DNA per cell in stationary phase populations. It seems certain, in any case, that increased radiation resistance cannot be correlated with increased DNA content. Also, phase microscopy of cells plated on microslide nutrient agar preparations indicates that neither multinuclearity nor multicellularity account for the higher survival observed in the radiation resistant E. coli populations.

If adequate concentrations of required metabolic precursors are supplied to cultures growing in synthetic medium, the rate of DNA synthesis during various growth phases parallels the rates of cell division and increase in viable number. Both DNA synthesis and cell division continue, though at a very slow rate, well into the stationary phase (at least four hours). The latter observation is inconsistent with findings reported elsewhere (Yoshikawa and Sueoka, Proc. Nat. Acad. Sci., 49:559, 1963) that DNA replication is completed by cells of a Bacillus subtilis culture entering stationary phase. However, preliminary experiments indicate that cessation, and perhaps completion, of DNA synthesis in stationary phase E. coli does occur if the supply of required precursors becomes limiting, either by exhaustion from the growth medium or as a result of developing culture conditions (e.g., lower pH) which interfere with the cells' capacities to utilize the available precursors.

The growth kinetics of cells in both logarithmic and stationary phases are particularly dependent upon thymine concentration. At any time during logarithmic phase, each successive exponential generation of viable numbers requires that the growth medium contain at least two- and-one-half times the amount of thymine which is to be incorporated into DNA during that generation. Lesser concentrations of thymine are detrimental to some portion of the population, and may result in changed sensitivity to radiation damage.

No detrimental effects are observed when RNA and protein synthesis are inhibited by uracil- and arginine-deprivation. This treatment of logarithmic cells was previously shown to force the cells into a metabolic state in which (1) they are indistinguishable from normal, early stationary phase cells and (2) their radiation resistance has increased to that observed for early stationary phase cells. It is a subject of current controversy whether this metabolically-induced condition (and the accompanying increase in radiation resistance) is related to a change in "state" of DNA (e.g., "orientation" of DNA synthesis to a specific terminal locus in all cells, such that all cells have a complete double complement of DNA, and initiation of new cycles of DNA synthesis cannot occur because the cells cannot make certain specific "initiating" proteins) or simply to a loss of capacity to synthesize DNA (i.e., DNA synthesis stops at random loci in the population as a

Using this flow system in conjunction with the electron spin resonance spectrometer, a study of the action of chemically generated free radicals upon model carbohydrates at room temperature has been initiated. The identification of such radicals characterized by their hyperfine interaction and measured lifetimes will be of great value in understanding the radiation chemistry of the nucleic acids.

The work described above is directly related to studies of correlations between damage and induced free radical activity in gamma-irradiated microorganisms.

Summary and Conclusions:

1. Bone Marrow Studies. These studies suggest the presence of a weak or absent histocompatibility barrier in the rat making it an undesirable species to study secondary disease. Recently reported studies of Malinin, and associates, raise the question as to whether a similar mechanism may not be operating in their experiments utilizing pooled guinea pig buffy coat. Although our studies have neither established nor negated the effectiveness of allogeneic marrow pooling as a method of modifying secondary disease, they do emphasize the necessity of rigorously controlled experiments in the study of this problem. Similar experiments utilizing other mammalian species are planned.
2. Canine Studies. Because this long-term study of post-irradiation lifetime clinical histories will not be completed for several years, no conclusions can be presented at this time.
3. Vaccine Studies. Final interpretation of data is incomplete at this writing; however, it seems clear that these studies show no differences in response to vaccination or challenge between irradiated and non-irradiated dogs.
4. Grid Shielding. Initial experiments have confirmed that animals irradiated through grids show less effects of radiation damage than unshielded animals exposed to the same doses. Preliminary studies also indicate that the protection afforded by grid shielding extends to very deep tissue.
5. Cell Culture Studies. Results from the radiation studies of L-strain rat fibroblasts have indicated that initial damage is the same in logarithmic- and stationary-phase origin cells and it is suggested that some extragenetic constituent having a key role in the control of cell division might be damaged by the radiation and that the initial concentration of this key constituent is higher in logarithmic than in stationary cells.

6. E. coli Studies. Studies of growth kinetics in relation to specifically altered pre- and post-irradiation metabolic events consistently show that changes, if any, in initial, primary, radiation lethal damage at the cellular level do not correlate with changes in observed survival. However, the results are consistent with the notion that observed changes in radiation sensitivity of the bacteria can be correlated with changes in certain biochemical processes. These biochemical changes result from environmentally induced changes in metabolic state of the cells, rather than from damage by irradiation. It is suggested that these biochemical processes are parts of the cells' "damage repair mechanisms". This notion is being tested by experimental and theoretical challenges.

7. Decomposition of Nucleosides. These studies are newly instituted; all data are incomplete; and therefore, no conclusions are presented.

ARMY RESEARCH TASK REPORT		REPORTS CONTROL SYMBOL CSCRD-6(R2)
ACCESSION NUMBER 37360	PROJECT, TASK, OR SUBTASK NO. RD 4-1-61 c 03.084	
1. REQUESTING AGENCY US Army Combat Development Command Nuclear Group, Fort Bliss, Texas	2. FUNDING AGENCY Defense Atomic Support Agency Department of Defense Washington, D. C., 20315	
3. CONTRACTING AGENCY NA	4. CONTRACTOR AND/OR GOV'T LABORATORY A Walter Reed Army Inst of Rsch Walter Reed Army Medical Center Washington, D. C., 20012 723-1000, Ext 3552	
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Woodward, Kent T., Lt Col, MC, Dir, Div of Nuclear Medicine WRAIR, WRAMC, Washington, D. C., 20012 576-2211 or Interdepartmental Code 198, Ext 2211 See Continuation Sheet		
6. TITLE OF: PROJECT <input type="checkbox"/> TASK <input type="checkbox"/> SUBTASK <input checked="" type="checkbox"/> Dosimetry of neutrons and gamma radiation (U)		
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964		
8. RESUME' (U) This subtask was designed to correlate known biological end-points with the physical dose of mixed ionizing radiation with the view of eventually obtaining a dose-dependent endpoint which may be used as a measure of effective total body dose. Known biological endpoints were to be used to correlate biological response to physical dose at the single organ and/or whole body level. Specific research efforts under this subtask were (1) neutron flux and gamma ray dose measurements in the reactor exposure facilities, (2) development of a technique for the measurement of neutron penetration by tissue activation, (3) detection and characterization of long lived radiation induced ESR signals in <u>in vivo</u> irradiated rat femurs, (4) establishment, procurement, and calibration of routine dosimetric techniques for use in the radiobiology research program utilizing the reactor, and (5) characterization of the ESR signal from commercially available tetrazolium salts. These studies have provided necessary information for reactor operations and for initiation of radiobiology research program utilizing the WRAIR Reactor. This is final report on this subtask.		
9. KEY WORDS Dosimetry, electron spin resonance, femur, formazan, gamma-ray dose, neutron flux, rat, tetrazolium, threshold detector, tissue activation, tissue equivalent.		
10. SUPPORTING PROJECTS Not Applicable		
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet	12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet	
<div style="display: flex; justify-content: space-between;"> DA FORM 1309R 1 Jun 63 PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of _____ </div>		

ACCESSION NO.

37360

ARMY RESEARCH TASK REPORT

13. PROJECT, TASK OR
SUBTASK NUMBER

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
R	D	4	1	-	6	1		C		0	3		0		8	4		

14. DATE OF REPORT (30-33)

30	31	32	33	34
0	6	6	4	4

15. SECURITY OF WORK (34)

16. TYPE OF REPORT

35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55
2																	0	6	6	3

17. SCIENTIFIC FIELD

 a. Topical Classific. (56-61)
 b. Functional Class (62-64)

56	57	58	59	60	61	62	63	64
0	4	0	1	0	3			

18. OSD CLASSIFICATION

(65-66)

19. R&D CATEGORY (67)

65	66	67
A	R	5

20. CONTRACT NUMBER

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
D	A															

21. GRANT NUMBER

28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46
D	A												G					

22. ESTIMATED COMPLET.
DATES

47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71
1	0	6	6	4	2					3					4					5				

23. PRIORITY (11-14)

24. PROGRAM ELEMENT
(15-26)

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26

25. CMR&D CODES

27	28	29	30	31	32	33	34	35

26. CDOG REFERENCE

 a. Paragraph No. (36-44)
 b. Functional Group (45)

36	37	38	39	40	41	42	43	44	45
									0

27. FUNDING

a. Est. Total Cost (11-15)

b. % Spent Intern. (16-18)

" " Extern. (19-21)

c. Total Obligation (22-26)

d. Progmd. Cur. FY (27-33)

e. " " " +1 (34-40)

f. " " " +2 (41-47)

g. " " " +3 (48-54)

h. " " " +4 (55-61)

i. " " " +5 (62-68)

j. " " " +6 (69-75)

k. Total Man Years of
Effort (76-78)

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
			8	2	1	0	0	2				1	7	9	

27	28	29	30	31	32	33	34	35	36	37	38	39	40
6	4				7	5							

41	42	43	44	45	46	47	48	49	50	51	52	53	54

55	56	57	58	59	60	61	62	63	64	65	66	67	68

69	70	71	72	73	74	75	76	77	78
								2	

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

- (A) Feldman, M. H., PhD, Dept of Biophysics
Div of Nucl Med, WRAIR, WRAMC, Washington, D. C., 20012
576-3014 or Interdepartmental Code 198, Ext 3014 49
- (A) Hightower, D., Lt Col, VC, Chief, Dept of Biophysics
Div of Nucl Med, WRAIR, WRAMC, Washington, D. C., 20012
576-3002 or Interdepartmental Code 198, Ext 3002 49
- (A) Lofberg, R. T., PhD, Dept of Biophysics
Div of Nucl Med, WRAIR, WRAMC, Washington, D. C., 20012
576-3014 or Interdepartmental Code 198, Ext 3014 49
- (A) Morton, P. W., 2/Lt, MSC, Dept of Biophysics
Div of Nucl Med, WRAIR, WRAMC, Washington, D. C., 20012
576-3015 or Interdepartmental Code 198, Ext 3015 49
- (A) Simpson, R. B., MS, Dept of Biophysics
Div of Nucl Med, WRAIR, WRAMC, Washington, D. C., 20012
576-3015 or Interdepartmental Code 198, Ext 3015 49
- (A) Smathers, J. B., MS, Dept of Biophysics
Div of Nucl Med, WRAIR, WRAMC, Washington, D. C., 20012
576-3015 or Interdepartmental Code 198, Ext 3015 49
- (A) Swartz, H. M., Capt, MC, Dept of Biophysics
Div of Nucl Med, WRAIR, WRAMC, Washington, D. C., 20012
576-3782 or Interdepartmental Code 198, Ext 3782 49

REPORTS. Final Report, Walter Reed Army Institute of Research
Annual Progress Report, 1 July 1963 - 30 June 1964.

Hightower, D., and Swartz, H. M.: Measurement of Neutron
Penetration by Tissue Activation. Proc. Sym. on Biological
Effects of Neutron Irradiations, sponsored by Intern. Atomic
Energy Agency, Brookhaven Nat. Lab., October 1963, in press.

Kilminster, D. T., Saccenti, J. C., McNeilly, J. H., and
Benck, R. F.: Neutron-Flux and Gamma-Dose Measurements at the
WRAIR Reactor. NDL-TR-57 (Nuclear Defense Lab.) December 1963.

Lofberg, R. T.: Some Observations on the ESR Signals of
Tetrazolium Compounds. In press.

Swartz, H. M.: Long-Lived Radiation-Induced ESR Signals in vivo.
Abstracts, Radiation Research Society, 12th Annual Meeting,
May 1964.

FINAL REPORT

Project No. DASA RD 41-61

Title: Biomedical (NWER)

Subtask No. 03.084

Title: Dosimetry of neutrons and gamma radiation

Description:

1. Neutron flux and gamma ray dose measurements were made in the WRAIR Reactor exposure facilities by the Nuclear Defense Laboratory.
2. A technique was developed which permits determination of neutron penetration in tissue without the addition of dosimetric devices or the use of tissue equivalent material.
3. Relatively stable electron spin resonance signals in in vivo irradiated rat femurs have been demonstrated and characterized.
4. Routine dosimetric methods to be used in the radiobiology research program utilizing the WRAIR Reactor have been initiated.
5. A variety of tetrazolium compounds from commercial sources were shown to possess an ESR signal.

Progress:

1. Flux Map. Subsequent to initial reactor criticality on 22 September 1962, considerable effort was exerted in bringing the Walter Reed Research Reactor into a fully operational and usable status. One aspect of this effort was to measure the neutron flux and gamma ray dose at some 60 to 90 points in the reactor exposure facilities. For the neutron flux measurements the threshold detector system consisting of the following detectors was utilized: Au, U-235, Pu-239, Np-237, S, Mg, and Al. Glass rods and plates were used for the gamma measurements. From the reactor operational standpoint analysis of the results of these measurements indicates that (1) experimental results are quite close to predicted values, (2) the intensity of radiations emitted from the operating reactor is linear with power, (3) the reactor as built is symmetric with respect to the center line.

From the dosimetric standpoint, a partial analysis of data from the thermal column region has been completed. A single collision neutron dose was calculated using the threshold detector system data. At 50 KW power operation, the total dose rate varies from 222 rad/sec at the innermost exposure position to approximately 4.4 rad/sec at the outermost, almost to the end of the thermal column. While neutron to gamma ratios from these data are probably not meaningful because the core was "cold", neutron to gamma ratios from the data vary from approximately 6.8 in close to the

bismuth to a high of approximately 18 further out in the thermal column. Measurements made in house also indicate that the neutron to gamma ratio changes with daily reactor operation. It appears that approximately 100 minutes are needed for this ratio to stabilize each day. The information thus obtained has provided sufficient information to satisfy reactor operations personnel and is a starting point for the dosimetry personnel.

2. Measurement of neutron penetration by tissue activation. A technique has been developed which is superior to the use of tissue equivalent materials and added threshold detectors in that (1) unmodified animal tissue is utilized in place of tissue equivalent material and (2) elements naturally occurring and distributed in tissue rather than added detectors which might cause flux perturbation. The technique might be viewed as two separate exposures of tissue at right angles to each other. The first exposure gives tissue activation as a function of depth scattering conditions and sodium concentration. The second gives activation as a function of sodium concentration only. Combined then, the effect of depth and scattering is obtained. To date, the experimental results represent relative sodium activation only, to the extent that the thermal activation cross section of sodium predominates. These results may be interpreted as thermal neutron flux at the point of interest. There is not distinction between incident thermal neutrons and those thermalized in the tissue. The next step, determination of actual dose gradient across tissue, is a large one and preliminary steps are just underway. Dog cadavers were prepared by clipping the hair from the thoracic and abdominal region. They were then placed in a deep freeze. Approximately two hours prior to exposure the dog cadavers were placed in liquid nitrogen. Freezing was necessary to stop any gross organ movement or diffusion of tissue constituents during or after exposure. Since the dog cadavers were not refrigerated during the actual exposure freezing to such low temperatures was required. Exposures were made at a reactor power level of 50 KW. Times for the exposures were varied between 30 minutes and one hour to give the desired degree of activation. Cadmium ratios were measured to give an indication of the spectral hardness. Rough approximations gave an estimated total neutron flux of 10^{12} in each exposure. After reactor shutdown the animal cadavers were removed from the exposure facility as soon as possible and returned to the liquid nitrogen. During the time required for recooling much of the short half-life activity decayed, thus reducing the exposure hazards to personnel. Sections of the dog cadaver approximately 1 cm thick were removed by making cuts normal to the spinal column in the long axis of the dog with a meat saw. Sections thus removed were used for two purposes: autoradiographs were made of some of the intact section, while other sections were cut into smaller samples for counting the induced radioactivity. The smaller samples were placed in small plastic containers and the induced Na-24 was counted. These initial counting data were of little value at this point since the activity of the samples was a function of depth scattering and unknown sodium content. The samples were held until the Na-24 had decayed to trivial levels and then reactivated along with appropriate sodium standards.

The samples and standards were counted as above and the sodium content of samples determined by comparing peak areas. The time corrected original data was divided by the sodium content of the samples. This gave experimental results in counts per gram of sodium, thus, relative sodium activation through the section was obtained.

Three separate and distinct experimental setups were utilized. This gave neutron spectra of different cadmium ratios. They were approximately 500/1, 10/1 and less than 1/10. Plots of the data thus obtained demonstrate there are obvious differences in the sodium activation through the dog. In addition, slopes of the curves were appreciably different when taken through the thoracic cavity and through the abdominal cavity. These results have demonstrated beyond any doubt the feasibility of using tissue activation for measuring neutron penetration.

Measurements are obtained in and from a true tissue environment. Approximations to geometric shapes are not required. Localized variations in tissue composition are automatically considered. Variation in exposure conditions can be detected. Three different neutron spectra were used and experimental results could easily be distinguished from each other. The fact that neutron penetration curves obtained from different sections of the body differ even when the impinging flux is essentially the same has been demonstrated. This is particularly important when one considers the actual dose delivered to specific internal organs, a fact which is frequently not fully appreciated.

Following development of the technique it was decided that additional induced activities in tissue might be studied. A preliminary study of those elements naturally occurring in tissues does not appear to be very fruitful, but other elements may be added by the intravenous route prior to destruction of the animal and then utilized. This would yield threshold data in depth and give a better understanding of spectral shifts and dose at specific points. It also appears that dose gradient across specific organs can be determined using this technique. This can be accomplished to any desired degree by reducing the size of the sample. The lower limit of sample size may well be of the order of cell dimension. The radiobiological significance of information of this type is quite exciting even in the speculative stage.

Even though the feasibility of using tissue activation has been established there remain several unknown factors of concern. The activation cross section of sodium follows the $1/v$ law fairly rigorously at the lower energies and is expected to continue to have a smooth function below room temperature. Experimental data on this point is lacking. However, inasmuch as we are dealing with the sodium activation at low temperatures we are conducting experiments to determine the sodium activation cross section down to 77°K. Another possible source of concern is the fact we are using frozen tissue and claiming results for normal tissue. Neutron absorption and

scattering should not be materially affected by the phase change but the experiments are being repeated with a dog exposed at room temperature and then quickly frozen.

3. Long lived radiation induced ESR signals in vivo. Long lived electron spin resonance signals were detected in rat bone irradiated in vivo and in vitro. In the in vivo experiments 200 gram white rats were irradiated whole body at room temperature (293°K) with Co-60 gamma rays at various dosages and sacrificed at various times after irradiation. At sacrifice, the long bones of the legs were removed, placed into liquid nitrogen, and examined in a Varian ESR spectrometer at 77°K . An asymmetrical ESR signal with g value approximately 2.003 was noted. The magnitude of the signal was a function of (1) the radiation dose and (2) the time interval between irradiation and sacrifice.

In the in vitro experiments, bone samples irradiated at 77°K gave a prominent spectrum consisting of several peaks with additional, partly resolved, fine structure.

Bone with the organic material extracted had signals similar to unmodified bone while bone with the inorganic material extracted had very weak resonances following irradiation. Bone irradiated at room temperature gave results similar to those observed in the in vivo experiments.

Ordinarily one does not see radiation-induced resonances in biological material at room temperature due to the rapid reaction of chemical species having unpaired electrons. It is suggested that resonances persist in bone because its highly organized structure offers many potential sites for electron and/or radical trapping. The persistence of such resonance in vivo is of interest both radiobiologically and dosimetrically - radiobiologically as an indication that not all the energy imparted to tissue is immediately converted into its final form, and dosimetrically as a potential after-the-fact indicator of dose in accidental radiation exposures.

4. Routine dosimetry. Study was given to the entire radiobiological dosimetry program which would be required for the research program utilizing the WRAIR Reactor. It was decided that knowledge of the following aspects of the exposure environment would be desirable: (1) total absorbed dose, (2) dose as a function of depth in tissue, (3) total neutron dose, (4) total gamma dose, (5) neutron dose to gamma dose ratio, (6) neutron energy spectrum, (7) gamma ray energy spectrum (8) total neutron flux, (9) dose rate. With the above factors in mind, the routine dosimetry methods were established. For total absorbed dose, tissue equivalent ionization chambers were fabricated by the Instrumentation Division, WRAIR. These chambers have now been calibrated and are in use. To determine depth dose the technique mentioned in paragraph 2 above was developed. In addition, phantoms have been used with the standard methods of dosimetry. Total neutron dose is

available by calculation using the threshold detector system developed by the Army Chemical Corps. While far from satisfactory, this is probably as good as is available at the present time. Total gamma dose is obtainable by the use of glass rods, lithium fluoride, and film. The latter two are in use at the present time, and additional calibration is being done on the glass rods. Experience in this laboratory indicates that lithium fluoride exhibits a thermal neutron response. This has not been reported from the manufacturer. It is under further study at the present time. Neutron dose to gamma dose ratio is obtainable by ratioing the above mentioned methods of measuring each independently. The neutron energy spectrum is obtained from the threshold data. This is very crude at the present time and studies are under way to see if this information can be improved. The gamma ray energy spectrum from the reactor in a radiobiology exposure condition is unobtainable at the present time. The total neutron flux may be approximated from the threshold detector data. Boron trifluoride tubes are also being considered as a method of measuring this parameter. No dose rate meters are presently in use. The dose rate is determined by dividing the total dose by the length of the exposure. Prior to this time two exposure facilities have been widely used. Most of the dosimetry has been done in one or the other of these. The first was into the beam catcher associated with port 5. Port 5 is a three inch cylindrical port. Measurements indicate that this beam is relatively homogeneous over its entire surface. It appears to be well collimated into the beam catcher. The other port which has been widely used is the thermal column door port. Several exposures have been made at this position. It was found after such exposures that the beam was relatively inhomogeneous across its surface. This has been proved both biologically and dosimetrically. Of interest is the fact that the radiation intensity across the port entrance on the inside of the thermal column door is fairly homogeneous. Some perturbation is occurring as the beam traverses the port in the door. This phenomenon is as yet unexplained but studies are underway to provide a better understanding of it. The routine dosimetry program now in existence is far from satisfactory as far as biological exposures are concerned, but it is a workable one and one which is being used in other laboratories so that results from WRAIR can be intercompared. It is hoped that the continuation of this program will result in better dosimetry data and a better understanding of the biological response to radiation exposure.

5. Tetrazolium compounds. A study was made of the electron spin resonance spectra of some commercially available tetrazolium salts and formazans. The origin and cause of the ESR spectra of 2, 3, 5 triphenyl formazan was investigated.

It has been observed that several commercial samples of 2, 3, 5 triphenyl formazan exhibit a complex ESR first derivative spectrum as the solid, and a nine-peaked spectrum in benzene solution. The cause for this unpaired electron signal was unknown and would not be anticipated from the normally accepted structural formula. Since this class of compounds have proved useful as radioprotective agents, radiation dosimeters and as

tracers in following enzymatic reactions, it was decided to investigate the origin and cause for this phenomenon.

A number of commercially available tetrazolium salts and formazans were studied for their electron spin resonance properties both as the solid and in benzene solutions, as applicable. The following substances yielded ESR spectra but not necessarily that of unpaired electrons: 2, 3, 5 triphenyl formazan, neotetrazolium formazan, tetrazolium violet formazan, tetrazolium violet, 2, 3, 5 triphenyl tetrazolium chloride, tetrazolium blue, nitro blue tetrazolium, p iodo nitro tetrazolium violet and neo tetrazolium chloride.

A more detailed study of the 2, 3, 5 triphenyl formazan was undertaken. Synthesis of the formazan by a variety of methods resulted in products with the signal. Careful purification of the synthesized product as well as the commercial product yield a signal free formazan. The signal was traced to an impurity formed during reductive preparation.

The ESR spectra of 2, 3, 5 triphenyl formazan was shown to be produced by an impurity probably formed during the synthesis. A variety of tetrazolium compound from commercial sources were shown to possess an ESR signal.

Summary and Conclusions: Work under this subtask has provided the necessary information for reactor operations and for initiation of the radiobiology research program utilizing the WRAIR Reactor. Techniques for the use of tissue as a dosimetric tool have been investigated and proven feasible. Further consideration of present dosimetric techniques has reemphasized the current shortcomings of such techniques. Additional research in this most important area will continue as an integral part of the radiobiology research program of this division. Refinement and extension of the techniques developed under this subtask using tissue may well provide better dosimetric information for future use.

ARMY RESEARCH TASK REPORT		REPORTS CONTROL SYMBOL CSCRD-6(R2)
ACCESSION NUMBER 37361	PROJECT, TASK, OR SUBTASK NO. RD 41-61 c 03.085	
1. REQUESTING AGENCY U.S. Army Combat Development Command Nuclear Group Fort Bliss, Texas	2. FUNDING AGENCY Defense Atomic Support Agency Washington 25, D. C.	
3. CONTRACTING AGENCY NA	4. CONTRACTOR AND/OR GOV'T LABORATORY A Walter Reed Army Inst of Rsch Walter Reed Army Medical Center Washington, D.C. 20012 723-1000, Ext 3552 <div style="text-align: right; border: 1px solid black; width: 20px; float: right;">49</div>	
5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER (P) Woodward, K. T., Lt Col, MC, Div of Nuclear Medicine WRAIR, WRAMC, Washington, D. C., 20012 576-2211 or Interdepartmental Code 198, Ext 2211 See Continuation Sheet <div style="text-align: right; border: 1px solid black; width: 20px; float: right;">49</div>		
6. TITLE OF: PROJECT <input type="checkbox"/> TASK <input type="checkbox"/> SUBTASK <input checked="" type="checkbox"/> Effectiveness of mixed radiation exposures (U)		
7. DATE OF REPORT DAY 30 MONTH June YEAR 1964		
8. RESUME' (U)Comparative mortality studies have been made for ICR strain mice receiving modified fission spectrum and 300 KV x-ray irradiation. Equivalent dose rates were designed at 15 rad/minute with a neutron/gamma ratio of 3.5:1 for the fission spectrum irradiation. Such mixed neutron-gamma irradiation revealed an LD _{100/30} of 500 rad with a mean survival of five days, whereas x-ray irradiation revealed an LD _{100/30} of 900 rad with a mean survival of twelve days. Serial sacrifices for histology and tritiated thymidine uptake are being done to evaluate gastrointestinal changes. The effect on skin of miniature swine is being studied with localized modified fission spectrum irradiation. Threshold and tolerance limits are being investigated and serial biopsies are being performed for histologic studies. Temporary epilation is seen at 350 rad. Permanent epilation with a moist epidermitis is found at 750 rad with subsequent healing. Partial body irradiation is being studied in beagle dogs with fission neutron and 300 KV x-ray irradiation at similar dose rates and graded total doses from 250 to 1000 rad. Detailed renal function studies are being determined as well as morphologic investigations including both serial renal biopsies and serial sacrifices for gross and histologic examination.		
9. KEY WORDS Modified fission spectrum irradiation, mice, miniature swine, beagle dogs, functional and morphological effects, skin, gastrointestinal tract, kidney, radiation biology		
10. SUPPORTING PROJECTS Not applicable		
11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet	12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES <input checked="" type="checkbox"/> NO <input type="checkbox"/> YES See Continuation Sheet	
<div style="display: flex; justify-content: space-between;"> DA FORM 1309R 1 June 63 PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of _____ </div>		

ACCESSION NO. 37361		ARMY RESEARCH TASK REPORT	
13. PROJECT, TASK OR SUBTASK NUMBER		11 22 23 24 25 26 27 28 29 R D 4 1 - 6 1 c 0 3 . 0 8 5	
14. DATE OF REPORT (30-33)		30 33 34 0 6 6 4 4	
15. SECURITY OF WORK (34)			
16. TYPE OF REPORT		35 36 47 48 49 50 51 52 55 3	
17. SCIENTIFIC FIELD a. Topical Classific. (56-61) b. Functional Class (62-64)		56 61 62 64 0 1 0 2 0 0	
18. OSD CLASSIFICATION (65-66)		65 66 67 A R 1	
19. R&D CATEGORY (67)			
20. CONTRACT NUMBER		11 12 13 14 15 17 18 21 22 26 27 D A	
21. GRANT NUMBER		28 29 30 33 34 35 36 38 39 40 41 45 46 D A G	
22. ESTIMATED COMPLET. DATES		47 51 52 56 57 61 62 66 67 7 1 0 6 7 0 2 3 4 5	
23. PRIORITY (11-14)		11 14 15 26	
24. PROGRAM ELEMENT (15-26)			
25. CMR&D CODES		27 29 30 32 33 35 27 29 30 32 33 35	
26. CDOG REFERENCE a. Paragraph No. (36-44) b. Functional Group (45)		36 39 40 41 42 43 44 45 0	
27. FUNDING a. Est. Total Cost (11-15) b. % Spent Intern. (16-18) " " Extern. (19-21) c. Total Obligation (22-26) d. Progrmd. Cur. FY (27-33) e. " " " +1 (34-40) f. " " " +2 (41-47) g. " " " +3 (48-54) h. " " " +4 (55-61) i. " " " +5 (62-68) j. " " " +6 (69-75) k. Total Man Years of Effort (76-78)		11 15 16 18 19 21 22 26 1 1 6 1 0 0 2 1 4 1 27 28 29 33 34 35 36 40 6 4 9 0 6 5 1 2 5 41 42 43 47 48 49 50 54 6 6 1 2 5 6 7 1 3 5 55 56 57 61 62 63 64 68 6 8 1 5 0 6 9 1 5 0 69 70 71 75 76 78 7 0 1 5 0 5	

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

- (A) Angel, C. R., Maj, MSC, Dept of Radiation Biology
Div of Nucl Med, WRAIR, WRAMC, Washington, D.C., 20012
576-3020 or Interdepartmental Code 198, Ext 3020 49
- (A) Casarett, G. W., PhD, The University of Rochester
School of Medicine and Dentistry, Rochester 20, New York
Area Code 716, Greenfield 34400, Ext 2178 30
- (A) Coburn, J., Capt, MC, Dept of Metabolism
Div of Med, WRAIR, WRAMC, Washington, D.C., 20012
576-3573 or Interdepartmental Code 198, Ext 3573 49
- (A) Hightower, D., Lt Col, VC, Dept of Biophysics
Div of Nucl Med, WRAIR, WRAMC, Washington, D.C., 20012
576-3002 or Interdepartmental Code 198, Ext 3002 49
- (A) Johnson, M., Sp5, Dept of Radiation Biology
Div of Nucl Med, WRAIR, WRAMC, Washington, D.C., 20012
576-3019 or Interdepartment Code 198, Ext 3019 49
- (A) Maier, J. G., Maj, MC, Dept of Radiation Biology
Div of Nucl Med, WRAIR, WRAMC, Washington, D.C., 20012
576-3001 or Interdepartmental Code 198, Ext 3001 49
- (A) McLaughlin, M., M.S., Dept of Radiation Biology
Div of Nucl Med, WRAIR, WRAMC, Washington, D.C., 20012
576-3012 or Interdepartmental Code 198, Ext 3019 49
- (A) Simpson, R., M.S., Dept of Biophysics
Div of Nucl Med, WRAIR, WRAMC, Washington, D.C., 20012
576-3015 or Interdepartmental Code 198, Ext 3015 49
- (A) Smathers, J., M.S., Dept of Biophysics
Div of Nucl Med, WRAIR, WRAMC, Washington, D.C., 20012
576-3015 or Interdepartmental Code 198, Ext 3015 49
- (A) Stromberg, L., Maj, MC, Dept of Radiation Biology
Div of Nucl Med, WRAIR, WRAMC, Washington, D.C., 20012
576-3023 or Interdepartmental Code 198, Ext 3023 49
- (A) Tessmer, C. F., M.D., The University of Texas
M. D. Anderson Hospital & Tumor Institute, Houston,
25, Texas 41

REPORTS. Annual Progress Report, Walter Reed Army Institute of Research, 1 July 1963 - 30 June 1964.

Semi-annual reports, DASA 78 are available from Defense Atomic Support Agency

Maier, J. G., Casarett, G. W.: Cellular Growth and Tissue Radiosensitivity: Tissue Studies in vivo and the Concept of Radiation Nephritis, Transactions, N. Y. Academy of Sciences, Vol 26, No 4, 1964.

ANNUAL PROGRESS REPORT

Project No. RD 41-61

Title: Biomedical (NWER)

Subtask No. 03.085

Title: Effectiveness of Mixed
Radiation Exposures

Description:

1. Mortality studies in mice have been done with fission spectrum irradiation and 300 KV x-ray. In addition, serial sacrifice has been made for histology of the gastrointestinal tract and epithelial regeneration studies with tritiated thymidine. Such studies are designed to elucidate the known sensitivity of the gastrointestinal tract to neutron irradiation as compared to x or gamma ray irradiation.

2. Fission spectrum irradiation has been given to the skin of the flank of miniature swine. Threshold and tolerance limits are being investigated by gross appearance and serial biopsies are being made for histologic evaluation.

3. Renal function and morphology are being investigated in beagle dogs subjected to localized irradiation of both kidneys with fission spectrum irradiation and 300 KV x-ray. Results are expected to give a measure of the relative damaging effect of fission neutron irradiation compared to x-ray at comparable doses and dose rates.

Progress:

1. Female ICR strain mice have shown an LD_{100/30} of 900 rad and a mean survival time of 12 days following irradiation with 300 KV x-ray at a dose rate of 15 rad/minute. Twenty animals per point were utilized with doses from 600 through 900 rad at 50 rad increments. Comparable studies with modified fission spectrum irradiation utilizing the Walter Reed Research Reactor revealed an LD_{100/30} of 500 rad with a mean survival time of five days. The modified fission spectrum source at the point of interest has a neutron/gamma ratio of 3.5:1 with a dose rate of 15 rad/min under the condition of administration. A special cylindrical graphite container was fabricated housing the individual mouse during irradiation. This graphite container was placed in the beam catcher in position at the north end of the thermal column of the Walter Reed Research Reactor by a lucite tube. The six inch square port for the fission spectrum irradiation was modified only by the graphite immediately surrounding the core and the permanent bismuth shield. There was no graphite in this portal in the thermal column per se. A series of animals at higher x-ray doses were irradiated to find

that dose causing a mean time of death of 5 days. This was found to be 2000 rad. Subsequently, a series of animals received 900 and 2000 rad x-ray and 500 rad fission spectrum irradiation with daily serial sacrifices preceded 24 hours by intraperitoneal injections of tritiated thymidine. Routine histologic sections for H & E staining and autoradiography of the stomach, duodenum, jejunum and ileum are in preparation.

2. Twelve miniature swine (Black African Guinea Hog) have received localized fission spectrum irradiation to the skin of the left flank by a three-inch diameter circular port of the Walter Reed Research Reactor. Two groups received a total dose of 350 and 700 rad respectively at a dose rate of 5 rad/minute with a neutron/gamma ratio of approximately 1:1. These animals are now eleven months post-irradiation. Temporary epilation was seen at 350 rad whereas a moist epidermitis was seen at 700 rad followed by gradual healing. Four serial biopsies have been taken on each animal as well as serial gross photographs. Histologic evaluation of these biopsies with routine and special stains is in progress. It is planned to sacrifice these animals at one year post-irradiation with a complete autopsy including gross and microscopic examination.

3. Long-term (up to six months) renal function and morphologic studies have been done on two groups of one-year-old beagle dogs following local irradiation to both kidneys. The first group (I) of ten male beagles had bilateral subcutaneous renal exteriorization and received modified fission spectrum irradiation. Two subgroups received 300 and 600 rads respectively to the mid-plane of each kidney. Serial renal biopsies were done at 2, 4, 8, 16 weeks post-irradiation. Control animals also had renal exteriorization and serial biopsies. All dogs in Group I were sacrificed at six months and histopathologic examination is in progress. The second group (II) of thirty female beagles received partial body irradiation to include the renal areas in two subgroups, (a) 300 KV x-ray with doses of 500 and 1000 rad to the kidneys, and (b) modified fission spectrum irradiation at doses of 250, 500, 750 and 1000 rad to the kidneys. Extensive pre- and post-irradiation function studies in Group II included excretory urograms, renograms with Hippuran-I¹³¹ and Renograffin-I¹³¹, urine and blood osmolality, timed urine flow following dehydration, blood creatinine, glomerular filtration rates, effective renal plasma flow, 24-hour sodium and potassium excretion following 4-day low salt diets. Serial sacrifices have been done at 2, 6 and 12 weeks in Group II with complete autopsies. Tissue sodium and urea gradients are being determined in the renal cortex and medulla at sacrifice.

Summary and Conclusions:

1. An RBE of 1.8 in ICR strain mice has been found using 100 per cent mortality at 30 days as an end point in comparing a modified fission spectrum irradiation of the Walter Reed Research Reactor with 300 KV x-ray. An increased sensitivity of the gastrointestinal tract is implicated and

further studies using histologic methods and autoradiography are in progress to further elucidate the injury and regeneration of the stomach, duodenum, jejunum and ileum.

2. Localized fission spectrum irradiation to the skin of miniature swine at a dose rate of 5 rad/minute has been found to produce temporary epilation at 350 rad and a moist epidermitis at 700 rad with subsequent healing. Serial biopsies for histologic changes are in progress.

3. Preliminary data indicate a greater renal effect from modified fission spectrum irradiation than x-ray irradiation in beagle dogs from both a functional and morphologic viewpoint. The determination of the magnitude of this difference must await completion of this subtask and extensive data analysis.

DISTRIBUTION:

CG, USA Med R&D Comd, Washington, D. C. 20315 (4)
Defense Documentation Center, Bldg No 5, Cameron Station,
Alexandria, Va. (20)
US AMedS Combat Development Group, BAMC, Fort Sam Houston, Texas
US Army Medical Equipment R&D Laboratory, Fort Totten, Flushing
59, L. I., New York
US Army Biomechanical Research Laboratory, WRAMC, Washington,
D. C. 20012
US Army Medical Research Unit, Institute for Medical Research,
Kuala Lumpur, Malaya
US Army Medical Unit, Fort Detrick, Maryland 21701
US Army Tropical Research Medical Laboratory, APO 851, New
York, N. Y.
US Army Medical Research Laboratory, Fort Knox, Kentucky
US Army Surgical Research Unit, BAMC, Fort Sam Houston, Texas
US Army Medical Research & Nutrition Laboratory, Fitzsimons
General Hospital, Denver 30, Colorado
US Army Medical Research Unit, Europe, APO 180, New York, N. Y.
US Army Medical Research Unit, Box 2011, Balboa Heights,
Canal Zone
US Army Research Institute of Environmental Medicine, Natick,
Mass.
US Army Institute of Dental Research, WRAMC, Washington,
D. C. 20012
US Army Aeromedical Research Unit, Fort Rucker, Alabama
Valley Forge General Hospital, ATTN: Librarian, Phoenixville,
Pennsylvania
Letterman General Hospital, ATTN: Librarian, Presidio of
San Francisco, California
William Beaumont General Hospital, ATTN: Librarian, El Paso,
Texas
Brooke General Hospital, ATTN: Librarian, BAMC, Fort Sam
Houston, Texas
Fitzsimons General Hospital, ATTN: Librarian, Denver, Colorado
Madigan General Hospital, ATTN: Librarian, Tacoma, Washington
Walter Reed General Hospital, ATTN: Librarian, WRAMC,
Washington, D. C. 20012
The Historical Unit, US Army Medical Service, Forest Glen
Section, WRAMC, Washington, D. C. 20012
United States Army, Europe, ATTN: Surgeon, APO 403, New
York, N. Y.
United States Army, Alaska, ATTN: Surgeon, APO 949, Seattle,
Washington
United States Army, Pacific, ATTN: Surgeon, APO 958, San
Francisco, California
United States Army, Caribbean, ATTN: Surgeon, Fort Amador,
Canal Zone
Technical Director of Research, Armed Forces Institute of
Pathology, Washington, D. C. 20315
CO, US Army Environmental Hygiene Agency, ATTN: Chief,
Library Branch, Army Chemical Center, Maryland

DISTRIBUTION (continued):

CG, United States Army, Hawaii, ATTN: Surgeon, APO 957,
San Francisco, California
CG, Eighth United States Army, ATTN: Surgeon, APO 301,
San Francisco, California
CG, 7th Logistical Command, ATTN: Surgeon, APO 612, San
Francisco, California
CG, I Corps Group, ATTN: Surgeon, APO 358, San Francisco,
California
CG, United States Army, Japan, ATTN: Surgeon, APO 343,
San Francisco, California
CG, United States Army, Ryukyu Islands/IX Corps, ATTN:
Surgeon, APO 331, San Francisco, California
US Continental Army Command, ATTN: Surgeon, Fort Monroe,
Virginia
First United States Army, ATTN: Surgeon, Governors
Island, New York, N. Y.
Second United States Army, ATTN: Surgeon, Fort George G.
Meade, Maryland
Third United States Army, ATTN: Surgeon, Fort McPherson,
Georgia
Fourth United States Army, ATTN: Surgeon, Fort Sam
Houston, Texas
Fifth United States Army, ATTN: Surgeon, 1660 E. Hyde
Park Blvd., Chicago, Illinois
Sixth United States Army, ATTN: Surgeon, Presidio of San
Francisco, California
Military District of Washington, US Army, ATTN: Surgeon,
Washington, D. C. 20315
First United States Army Medical Laboratory, 90 Church Street,
New York, N. Y.
Second United States Army Medical Laboratory, Fort George G.
Meade, Maryland
Third United States Army Medical Laboratory, Fort McPherson,
Georgia
Fourth United States Army Medical Laboratory, BAMC, Fort
Sam Houston, Texas
Fifth United States Army Medical Laboratory, St. Louis Area
Support Center, U. S. Army, 12th and Spruce Streets, St.
Louis, Missouri
Sixth United States Army Medical Laboratory, Fort Baker,
California
CO, 406th Medical Laboratory, United States Army Medical Com-
mand, Japan, APO 343, San Francisco, California
Air University Library, ATTN: AUL3T-62-377, USAF, Maxwell
Air Force Base, Alabama
Library, National Institutes of Health, Building 10, Room
5N118, Bethesda, Maryland 20014, ATTN: Acquisitions Section
National Library of Medicine, Bethesda, Maryland 20014
Director, US Army-SEATO Medical Research Laboratory, APO 146,
San Francisco, California
Chief, Life Sciences Division, Army Research Office, 3045
Columbia Pike, Arlington, Virginia

DISTRIBUTION (continued):

Biomedical Library, The University of British Columbia,
Vancouver 8, Canada
Assistant Army Attache (Medical), U. S. Navy 100, Box 79,
FPO, New York, N. Y.
A. S. Benenson, M.D., Director, Pakistan-SEATO Cholera
Research Laboratory, Institute of Public Health,
Mohakhali, Dacca-5, East Pakistan
Chief, US Army Medical Research Team (WRAIR) Vietnam, United
States Army Support Command Vietnam, Drawer 100 (Medical)
APO 143, San Francisco, California
Director, Armed Forces Radiobiology Research Institute,
Bethesda, Maryland 20014